COVID-19
Virus and Vaccines

G. G. Bolich, PhD

COVID-19 Virus & Vaccines.

G. G. Bolich, Ph.D.

EVS PRESS
2022

EVS Press

Spokane, Washington

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For Everyone Who Wants to Understand the Vaccination Choice They Make

Publisher Cataloging-in-Publication Data

Covid-19 virus and vaccines.
Bolich, G. G. (1953-)
First e-Book edition.
1. Vaccines 2. Public Health
I. Title
QR189.5.B65 2021
614.47.B689—dc23

Acknowledgments: *Note Bene*

Grateful acknowledgment *is made to the people who crafted the illustrative material for United States government organizations like the National Human Genome Research Institute (NHGRI), the Centers for Disease Control and Prevention (CDC), and the National Institute of Allergy and Infectious Disease (NIAID), all organizations from which some of the images used herein had their origin. This public domain material is an invaluable resource for picturing structures and processes important to understanding the matters discussed in this volume. Elements of some of these illustrations are incorporated into other illustrations; acknowledgment of the origin of these elements is made here Similarly, grateful acknowledgment is extended to those scholars and scientists who have graciously made their work available through Creative Commons Attribution (CC BY) license. These are acknowledged in place in accord with that license and those notices indicate any alterations made.*

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Cover by BachsCrafts

Printed in the United States of America
1st edition

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About the Author

G. G. Bolich might be called a 'professional scholar,' trained in research, critical thinking, and objective reporting across multiple academic disciplines. In preparation for such a career he earned six degrees, five of them advanced ones, including two doctorates.

His career has spanned more than four decades in higher education, teaching at both undergraduate and graduate levels. His second PhD focused on biological psychology (sometimes called 'psychobiology' or 'medical psychology'), including advanced work in neuropsychology and culminating in a dissertation on the neurobiology of psychological boundaries. As a university teacher he taught graduate level courses in psychopharmacology and various courses related to research, including that involving genetics and various medical matters.

But his research interests have extended far beyond the boundaries of his assigned courses. The author of dozens of articles and more than 40 books, he has published work in a number of fields, including medicine. Previous works have examined matters involving subjects covered in this present volume, such as genetics. In all these works a predominant theme has been the encouragement of further research by readers. His writing intends to demystify complex concepts and intricate research so that it is accessible to all who are interested in learning and expanding their knowledge.

Please note that this present book is a complementary volume to the previously published *Understanding COVID-19 Vaccination Arguments*, although it is not necessary to have read that book before using this one.

About EVS Press

Ecumenical Ventures in Scholarship (EVS) *Press* began, as the first word in its name might suggest, with the express interest of reaching across religiously diverse lines to offer sensible, nonpartisan scholarship. It was hoped such would offer common ground for people from different perspectives, providing them material they could trust as rational and evidence-based, and raising topics for fruitful discussion.

This same basic interest has continued, though the Press has ventured into other areas of interest. This title, for example, aims to provide the same sensible, nonpartisan scholarship on a topic of widespread interest in society today. The fact that the areas covered—virology, medicine, immunology, and vaccinology—are all grounded in science only furthers the basic aim of relying on rational, evidence-based discussion.

In the future, EVS Press will continue striving to offer to interested nonspecialists and specialists alike dependable and accessible materials on important topics.

Introduction

Like my previous book, *Understanding COVID-19 Vaccination Arguments*, this is a volume I did not want to write. But as in the case with that earlier book, I felt compelled to do what I can to alleviate the anxiety that fuels so much vaccine hesitance and resistance. That first book on the subject taught me that in most instances anti-vax sentiment is not genuinely fueled by concern over science, for the vast majority of anti-vaxxers, including anti-vax doctors, show either profound ignorance of the science or misrepresent it in the service of their far greater concern over certain cultural values and beliefs, or in the interest of personally profiting from the pandemic. In that earlier volume I let both sides—pro-vaccine advocates and anti-vaxxers—speak for themselves before weighing their arguments. I also appealed to certain ethical and cultural convictions shared by both sides as a way toward more fruitful discourse between them.

Regrettably, I am not optimistic that either side cares to hear the other side, let alone understand their opponents—but that cultural divide found in so many arenas of society is a topic for another day (and my forthcoming *American Plague: The Culture of Belief*). Here my address is to the especially interested nonspecialist—the general reader who is not intimidated by wrestling with science because she or he understands that even a modest effort can yield significant results.

As mentioned above, this book is a complement to my earlier one, though with an even narrower focus. In that volume I devoted a mere handful of pages specifically to the virus that causes COVID-19 and to exactly how vaccines work. That material is vastly expanded here. Whereas that earlier book presents the *arguments* around COVID-19 vaccination as offered by vaccine advocates and as disputed by anti-vaxxers, it does not itself spend much time on explaining the nuts-and-bolts science of how the coronavirus is built and operates, nor the same for the vaccines approved in the United States.

Because the science of such matters is often reduced to supposedly authoritative declarations so short and simple they amount to no more than sound bites for the evening news, the extent of many people's knowledge of the virus causing COVID-19 and the vaccinations meant to counter it remains dismally limited. For many folk it has become a choice between advocates on one side shouting, "The disease is deadly, the vaccine safe, take it!" and anti-vaxxers shouting even louder, "The disease isn't that much of a risk, the vaccine isn't safe, don't take it!" No wonder so many hesitate.

As with my other book I am not optimistic that at this point many in the general public have either the interest or the patience to follow the science even though doing so will not merely increase understanding but alleviate much of whatever anxiety may linger over receiving vaccination. Yet even if my sense of the American mood is right, I am convinced that even the few who are desirous of such a work at this time are worth attention, and that this volume will serve in future as a sort of historical record that helps others looking back to understand the science as it exists when this was written.

I am neither a virologist nor an immunologist (or any sort of medical doctor)—and that may be a good thing. I am not burdened by their training to communicate in a certain fashion relying exclusively on the technical jargon of their disciplines. As a professional 'outsider' I don't carry whatever biases may be implicit from such a background, nor the same philosophical convictions. My independence, like that of the readers to whom this is addressed, is that of someone who wants to profit from the immense learning and contributions of these professionals without actually becoming one of them.

Accordingly, *I write as a reader*, a member of a lay audience of nonspecialists, who wants to understand the science and needs it presented in a manner that can be readily grasped without advanced degrees. At the same time, like my intended readers, I want to read something as trustworthy as it is readable. I want to know it is not merely backed by reputable science but faithfully reports it. Although by the standards of scientific publications this book is rather lightly sourced, it still cites hundreds of scholarly books and articles—all of which offer additional information and point beyond themselves to even more research for those desirous to continue pursuing the subjects covered in this volume. This volume also faithfully repeats scientific language, with all its Greek- and Latin-derived terms, and many, many abbreviations.

That said, I promise to keep us in the shallow end of the deep science pool. Please note: this is *not* the wading pool or kiddie pool; it is a full Olympic-sized pool, though grading from a shallow end to a deep end for diving. We are mostly staying in the shallows, and if there arise any doubts concerning that, simply consult any of the scientific sources drawn upon and see how shallow we truly have remained! I can't, however, promise that we won't at times be standing on our tip-toes. Science may make progress incrementally, but it gets deep fast enough. Put bluntly, this is a book reporting scientific research and it is not *easy*.

The fact is that the science covered here is almost all material most of us aren't familiar with; the abbreviations, vocabulary, and concepts will be strange at first. If anatomy and physiology are challenging enough on their own, relating them, as in speaking of the structure and function of cells and their biological processes, is especially challenging—and so what is here will to the scientist often appear as surface level explanation. But the goal is not to understand the science as a scientist might but to grasp enough of what a professional knows to achieve a basic sense of the virus, immune system, and vaccines. Most of the articles cited in these pages are very technical and the information used from them typically comes from the more basic introductory portions of the material. But these articles are still useful to pursue independently, both to build a more refined understanding and to examine the sources those authors used, which can lead to still further avenues of learning.

I understand that the language found in those articles—and within these pages—is often challenging. But the vocabulary of science intimidates many people quite unnecessarily. Like any other unfamiliar area in life, it takes some exposure, patience, and guidance to learn enough to ease anxiety and build to-

ward a comfortable level of understanding. I have been selective in how much of the scientific language is reproduced, and I have taken pains to break it down. For example, not infrequently I will offer more common language followed by a word or phrase in parentheses, with quotation marks around it. This word or phrase thus marked is the term found in scientific literature, which I have 'translated.' For example, I might say something is 'diverse' and follow that word with this: ('heterogeneous'). I have avoided the depth of detail an ordinary reader neither needs nor wants. To grab hold of the science about COVID-19 aimed at here does not require becoming a virologist, epidemiologist, microbiologist, immune system scientist, or medical doctor. But it does require some *patience* over time. This is not a volume to be read in one sitting.

I am, fortunately, well-positioned to read and understand the professional specialists and report their work. Unlike some writers who reach beyond their level of competence, I have stayed carefully within mine and drawn heavily on others whose expertise exceeds my own in various matters relevant to understanding the subject at hand. Of course, like anyone else, I am capable of mistakes and it will doubtless be a good idea to attribute errors to me rather than my sources. More importantly, checking the sources themselves and wrestling with the technical literature is the best way to grow in understanding—and to check whether I have gotten that science right. Understandably, because scientists usually write to other scientists in the same field, scientific literature is filled with technical terms and jargon—and sometimes written by folk for whom English is a second language. While I concentrate on rendering things into more ordinary English, I do not want to convey that all the scientific literature is impenetrable. Much is relatively easy to read and follow. Toward that end I do occasionally quote directly from various scientists to demonstrate that what they write often is reasonably accessible to those of us who are not experts.

If I may have made you a little uneasy, let me tag on some further reassurance. Both my educational background and the demands of subsequent professional obligations have resulted in a thorough acquaintance with medical history, terminology, and research. Some of this acquired learning has been previously published in various scientific and medical writings, including my book *Understanding COVID-19 Vaccination Arguments*. Both within and outside classrooms I have spent a good amount of time communicating sophisticated scientific studies to nonscientists. This volume reflects how I have learned to do so.

This book aims to address the widespread poverty of understanding of the virus and the vaccines. Further, it intends to do so in a manner that uses easily comprehended metaphors, repetition, illustrations and other techniques to help bridge between the vocabulary of science and the language of ordinary life. In effect, I am offering here a *translation* of science. Clearly that means, as all translation work does, that I am choosing *how* to present material—relatively simply but hopefully not simplistically—and *what*, out of the thousands of studies on these matters, to offer. My choices are guided by several considerations: first, to expose as wide a range of literature as is feasible in a work of this size and na-

ture; second, to select materials at varying levels of difficulty, and finally, to present materials I think both interesting in themselves and relevant to this book's goals. While I favor scientific journal articles I also dip into some basic textbooks to draw attention to them, and I use news sources particularly for interview materials. Please understand that because so much basic material is routinely repeated across sources, in some respects the citation of particular sources is almost arbitrary as it merely means this source, too, is recounting well-accepted information. My choice of sources thus often intends to document the information I've reported while also pointing to a source that may be interesting in its own right because it proceeds to add more depth or explore something I think worth looking at. If some choices seem idiosyncratic, so be it.

It is easy enough to see that this is a lengthy book despite its promise to stay in the shallow end of the pool. The length, though, makes sense. Each subject—virus, immune system, vaccines—could easily fill a hefty volume by itself. They require time to cover adequately, and the abundant use of illustrations adds to the length considerably. But the length actually aids comprehension because the illustrations, repetitions, and other features that create this length also contribute to mastering the contents. Still, all that is needed or aimed at here is achieving a basic grasp sufficient to experience a warranted confidence that the nature of the coronavirus that causes COVID-19 makes sense. Then, building on this sense about the virus, grasping how the vaccines work will help make sense of how the two parts fit together. Between them is the medium of the human immune system, which the virus provokes and the vaccines intend to prepare for the fight.

The Plan of the Book

The book may be looked at as having two broad divisions book-ended by an introduction and conclusion. Put simply, the book is about the enemy—the COVID-19 virus—and our efforts to defend against it. The introduction precedes four chapters on the enemy, and then four chapters on our defense follow, with a concluding chapter wrapping up the book. Looked at a second way, as possessing three parts—the virus, the immune system, and the vaccines—may offer a little more clarity about the content. Either way, the structure is intended to provide a logical organization for looking at our subject.

The first three chapters move from a broad consideration of viruses through an introduction to coronaviruses to a specific focus on the coronavirus that causes COVID-19. The next three chapters then look at the disease and the immune system's responses. The final part of the book again proceeds from the broad to the narrow by examining vaccines in general before looking at the three approved vaccines in the United States. To this tripartite structure has been added an epilogue looking to the future.

In each part I offer the vocabulary of science, break it down into more digestible form, provide metaphorical translations to better grasp the meaning, and set out illustrations to help visualize the matter at hand. I don't hesitate to

repeat terms and their meaning in different chapters both because some people skip around when they read and because repetition is a key to learning. For those who find this tiresome such repetitions can be skipped over. In translating scientific language something may be 'lost' because science terminology aims at a precision often lacking in normal discourse. In simplifying scientific articles it can be easy to somewhat oversimplify; to whatever extent that happens here no fault should be laid at the feet of the scientists but placed squarely on me.

The first chapter, "Viruses," is foundational to the rest of the book. In it is a general, even somewhat roundabout discussion of what viruses are and what is needed to understand discussion about them. That latter part of my statement is crucial; chapter one presents some basic biological information that extends well beyond the strict topic of viruses but is necessary for grasping portions of the discussion later in the book. Thus we begin with some basic genetics, talking about DNA and RNA. For those who have been exposed through school to such material it may be tempting to skip over this material. I urge you not to do so. If it proves nothing more than a review, at least it is a quick one—and I trust an easily accessible one. In addition to this foundational material—all richly illustrated—there is discussion of such questions as whether viruses are actually alive, how small they actually are, and what such matters may mean for understanding them.

The second chapter, "Coronaviruses," narrows our scope to a particular family of viruses. It is no exaggeration to say this family has received the most attention by the general public and by professionals in the 21st century of any virus family. That is because certain family members have proven to be the causal agents of deadly diseases, including the current COVID-19 pandemic. One anti-vax claim has focused on concern over how quickly vaccines against COVID-19 have been developed so understanding our history of studying this virus family is an important part of responding to that concern and opens our discussion. In this chapter the same kind of things covered in chapter 1—e.g., genetics, protein structures and functions—are covered. Though the chapter aims chiefly at examining what the coronaviruses share in common, some differences among them exist and are noted. With respect to such differences the attention is on the human coronaviruses. In fact, the more specific the chapter becomes the more the human coronaviruses are in view. The chapter concludes by summarizing the big family picture, showing how its members are grouped, and offering some short coverage of the human coronavirus family members. This leads naturally and directly to our special focus on the most notorious member of the family—the virus behind COVID-19.

The third chapter, "The Enemy: SARS-CoV-2," brings focused attention to the specific coronavirus that is the cause of COVID-19. If the previous two chapters have been read, this one will make more sense. However, anticipating that some readers will start with this chapter, there is enough repetition of key points to help keep one on track. In it the specific biology of the virus is presented (with illustrations). The chief difference between this chapter's presenta-

tion and the previous one is an effort to highlight the aspects of SARS-CoV-2 that get the most attention, and explain why this attention matters. At the very end of this chapter a summary illustration captures the heart of the discussion, which has been the tracing of the six stages of the viral cycle.

The fourth chapter, "COVID-19," focuses on the syndrome caused by SARS-CoV-2. After briefly discussing the meaning of the name and history of the disease, attention turns to a logical sequencing of matters associated with it. First covered is how the disease is transmitted. Next comes an extensive examination of infection, including likelihood of being infected, factors related to individual susceptibility, the course and duration of infection, and disease progression. Then an examination of symptoms associated with the disease is presented, followed by a section on special conditions, such as 'long COVID.' The chapter is rounded out by how COVID-19 cases are classified and how testing is done to confirm cases. An emphasis is placed throughout on explaining underlying biology, which helps tie this chapter with the others.

The fifth chapter, "The Human Immune System," is in some ways a step back into more general matters and a step forward into the territory between the virus and the vaccine response. Accordingly, with respect to more general matters the chapter explains the nature of the immune system, its component parts and structure, and how it works. Of course, as with all other matters in this volume, the material stays in shallow water with brief descriptions of most matters and an aim to assist in simply acquiring a basic grasp of the immune system. Even so, this chapter is both long and at points difficult unless one has a background already in immunology. Treatment of the many structures and functions of this complex system may be frustratingly brief, but footnotes point the way to further exploring the various matters.

In the sixth chapter, "The Immune System vs. the Virus," is found the material more specific to COVID-19. This chapter is actually longer than the preceding one even if it presumes it. The focus can be tighter since there has just been a review of the immune system to provide a context for the application to how it responds to SARS-CoV-2, but the chapter is longer because this application leads into areas that also relate to the chapter on COVID-19 and focus on matters of special interest, such as the debate over whether natural immunity provides better protection than vaccination. A large section near the end of this chapter examines closely this topic. That discussion sets the stage for the final part of the book.

The seventh chapter, "Vaccines," presents general information about the logic, purpose, and nature of vaccines. This is accompanied by a brief review of the history of vaccination. Then attention is given specifically to the issue of developing vaccines for COVID-19. It varies from the chapter that follows it by offering a much broader examination of vaccines. This chapter is the one most like my previous book on COVID-19 because it addresses anti-vax arguments and the concerns of the vaccine hesitant, and repeats some of the material found there.

The eighth chapter, "Three Vaccines," first details the mRNA vaccines, those produced by Pfizer and Moderna. Next follows an examination of the Johnson & Johnson vaccine. While providing the same kind of examination as for the mRNA vaccines, it also specifies how this vaccine is different and addresses the specific concerns raised about it. In this chapter the vaccines are also compared with another in light of research about their similarities and differences, strengths and vulnerabilities.

The ninth and final chapter, "Epilogue: Lessons from a Pandemic?" quickly reviews why the anti-vaccine movement has succeeded—much like a virus itself—and what needs to happen before the next pandemic arrives. It specifically addresses the question being raised in early 2022 as to whether the COVID-19 pandemic could be said to be over with the virus moving into an endemic status as something that will have to be lived with like a seasonal virus.

In reviewing this lineup, it might be wondered why both coronaviruses as a group and the coronavirus that causes COVID-19 are included as separate chapters. There are at least three good reasons for this. First, the general chapter provides broad context for the more specific treatment of the following one. Second, offering an overview that looks at the main features of this virus family both structurally and functionally sets the stage for an even more focused approach in chapter 3, with the added benefit of enough repetition to aid mastery of the content. Finally, if we are realistic we must acknowledge that coronaviruses are likely to continue to grab attention in the years to come. Three deadly coronaviruses since the start of the still young 21st century should tell us that we better do our best to understand the whole family.

Now, as we wrap up this Introduction, some quick notes about some other matters. This book has many illustrations. As noted in my acknowledgments many come from materials placed in the public domain, or made available by Creative Commons license, by different agencies, publishers, or authors. I have adapted many illustrations for the purposes of this book (and noted such changes). Some of the illustrations are my own. I am deeply indebted to all those who, far more skilled than myself, have lent their talents to helping the rest of us better understanding the matters presented. In addition, while it may or may not be an aid to memory, the boxed material is color coded by chapter. It is important to remember that most illustrations are not intended to provide realistic portrayals of exceedingly small pieces of matter or their interactions. They are, instead, artistic representations intended to help visualize in broad and basic ways various structures and processes.

Although it is traditional in scientific literature to use *end* notes rather than *foot*notes, and a somewhat different citation and bibliographical format, my choices in these matters has been guided by my aim to reach a nonscientist audience. It seems desirable to see immediately what source may be cited or, as is sometimes the case, find a brief explanatory note that may help clarify the main text. The chief cost of this strategy, of course, is that numerous footnotes can eat up space on a page and potentially slow a reader down.

Finally, please remember that scientists—and the rest of us—are learning more about COVID-19, the virus that causes it, and its vaccines, all the time. This is simply a report-in-progress, a statement reflecting some of what is known, or thought, at the present time. It will remain vitally important not to presume what is said here is definitive; as facts on the ground emerge, ideas must change or learning ceases. Therefore, it is my hope every reader will continue to monitor these matters in an informed and thoughtful manner, resisting irrational fears persistently being set forward by others who refuse to exercise the same diligence as those who come to a book like this one.

About the Illustrations

Some reference has been made already to the illustrations found in this volume. Please be sure to see the acknowledgments at the start of the book, but I wish to remark here more on them. My artistic skills are limited enough I tell people I can't draw a straight line even with aid of a ruler. Nevertheless I have ventured to create many of the illustrations in this book, not infrequently borrowing and repurposing elements of illustrations made available for public use by groups such as the National Human Genome Research Institute (NHGRI) or the National Institute of Allergy and Infectious Disease (NIAID). I am especially indebted to the NHGRI for illustrative material related to genetics and to NIAID for illustrative material related to the immune system. The material used is acknowledged in place the first time it appears, but sometimes elements (for example, cell illustrations) are then repurposed in later illustrations without explicit acknowledgment in place, trusting that a reader will remember these elements from previous use. This is done simply to avoid cumbersome repetition of credits that might detract from the illustration, which is a composite piece.

Every effort has been made to verify all materials used as either outrightly placed in the public domain (e.g., because created by government employees for uses explicitly rendering them such), or made available under Creative Commons (CC BY) license, which allows reusers like myself to distribute, remix, adapt, and build upon the material in any medium or format, so long as attribution is given to the creator. The license allows for commercial use. I happily make all my own illustrations available as such. The nature of science is to *share*. I am especially grateful to scientists who in their journal publications have been gracious enough to make their material available by CC BY license and such work is thoroughly acknowledged in the places where such material is used.

Finally, I also am indebted to those who granted me permission to display their work for this educational endeavor. Even in a world where so much competition exists and people often have commercial ends in mind, it remains part of the core nature of science to collaborate and disseminate the results of long hours of often tedious research. I have been inspired in my own illustrations by the careful explanations and alternative illustrations offered by many other scholars. Even when their work has not been used here it has informed my understanding and contributed to my presentation.

A more audacious author than myself might tell a reader how to read a book, but I am all in favor of anyone reading this however they wish—and to whatever extent. Nevertheless, I am bold enough to suggest there are various ways that might prove beneficial. In that spirit, here are a few suggestions.

First, *reading the book in order from beginning to end* is a good choice, but not always the most practical. I designed this book with a definite order of contents in mind, and structured it in a very definite manner. So reading straight through will provide first a background in viruses, then an overview of the coronaviruses, followed by an examination of SARS-CoV-2, the virus behind the COVID-19 pandemic. So the material starts broad and gradually becomes more specific. This same process is used within each chapter. It seems logical after studying viruses to examine the disease—COVID-19—that prompted writing this book. By placing this material where it is anyone who has been reading straight through is best prepared to see the disease in its biological context. Then the book pivots from the offensive nature of an attacking pathogen to the human defense mounted against it. That entails first examining the human immune system and then looking at vaccines—a human effort to assist Nature. As in previous chapters the material proceeds from broader matters to more detailed ones—both in the sequencing of chapters and within each one. Thus the patient reader is rewarded by tracing the threads that weave through the whole work and bring it unity.

But that is a lengthy process and the most demanding one. For some readers it won't be a very practical option.

So, *treat the book as a reference tool*. In this way it is not necessary to work systematically through the whole—at least not all at once. Instead, select the matters of most interest or concern. Topics can be readily discerned from the detailed Table of Contents. Don't worry about not being able to grasp something because the rest of the book hasn't been read; a dose of repetition, some cross-referencing, and a liberal use of simplifying explanations should suffice. In this manner, too, the illustrations can be helpful.

In fact, a third way to read this book is simply *trace the gist of material on a subject by starting with the illustrations*. They can provide at least a foothold to anchor a rudimentary grasp of a wide range of topics. They always occur next to descriptive text which they are meant to help represent pictorially and thus aid in understanding it. This procedure can be paired with another very useful one.

A fourth way to proceed is to *use the built in level tools—the titles and subtitles*. Just following the organization of material can help master it. For example, in the chapter on the immune system the subtitles show that the immune system is a two-in-one system. The more fundamental is the innate immune system, which is described in both structure and function—all indicated by subtitles. When a section like that titled *Three Critical Defensive Immune System Actions* is reached the subtitles make it plain what each action is. Thus, for instance, the

first action is subtitled **1. Recognize the Danger**. That section then has smaller subtitles naming the parts of the immune system that recognize danger and then naming what the dangers are that need to be recognized. Focusing on subtitles is a sensible way to help remember the structure of content and key terms. When coupled with illustrations, one is in great shape to remember basic things.

Finally, *avoid getting bogged down*. My definition of 'the shallow end of the pool' may be different from anyone reading the book. For example, discussing the immune system—a complicated matter—required making decisions about how much and what to present. Some will find the coverage frustratingly brief and inadequate, while probably more will find it irritatingly detailed and hard to understand, even with illustrations. Stay fixed on the goal of gaining the level of knowledge and understanding desired or needed. If that means more than what this book provides, great!—that is why the footnotes point in directions for further study. If the content in a section or chapter is too detailed, rather than getting disgusted or discouraged, skim or skip what doesn't seem essential.

Chapter 1
Viruses

What Is a Virus?

Viruses are puzzling entities. I use the term 'entities' advisedly, because a basic and as yet unresolved question is whether viruses are living organisms or not. Most people think of them as living, but microbiologists are a lot less confident on the matter. While it can be challenging for us to set aside preconceived notions, our goal in this volume is to think like a scientist, to see matters through the lens of science. So let's act like a scientist, starting with a basic question and then proceeding from observed facts to offer a description.

What is a virus? Even for a scientist that's a question that can be answered at varying levels of detail. For example, let's hear an expert in genomics and molecular biology offer a succinct description:

> A Virus is a small electron microscopic parasite, incapable of reproducing by its own, survives by directing the host cell machinery for the production of more viruses, which emerge from their respective host cell through lysis. . . . . Viruses are small submicroscopic, obligate intracellular parasites, which contain either DNA or RNA as genome protected by a virus-encoded protein coat called capsid.[1]

Any reader who readily comprehended all of that description is hereby excused from reading the remainder of this chapter, because I am going to presume most readers need a simpler start. So let's back up a moment.

Whether viruses may be judged as 'living' or not, as a biological entity they are the most abundant on earth—some 10 times the number of bacteria. New viruses are still being discovered. The one that causes COVID-19 was unknown until it burst onto the scene in such a frightening fashion. Although more than 4,000 viruses have been identified—arranged into 71 'families'—this represents a very small fraction of the number estimated to exist.[2]

More significantly for most of us, viruses can cause serious illness and death, which is why most of us pay attention at all to them. If COVID-19 was an inconsequential illness on the level of the common cold very few people would be talking about it or reading something like this book. The fact that viruses are so small they can't be seen yet so powerful they can bring death prompts anxiety; viruses represent an unseen, potentially deadly danger.

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[1] Chaitanya, *Genome and Genomics*, 1. English is not Chaitanya's first language, which accounts for some awkwardness of expression. To keep that from unduly interfering with reading and thus become an unneeded distraction I have corrected the printed version's 'emerges' and 'contains' by dropping the 's' to conform to standard English conventions.

[2] Chaitanya, *Genome and Genomics*, 3, puts the number of estimated viruses in the earth's atmosphere alone at 10^{31}–10^{32}.

Knowledge offers a degree of control by reducing what is unknown. So let's break down what our virus expert has said and reduce what we don't know.

First we are told that viruses are small—so small they are submicroscopic and require an electron microscope to see. Although we are far from having identified all the viruses that exist, what we have seen at present is that in scientific measurement terms their size is described by tiny bits of genetic material—"base pairs," that is, a portion of genetic material found in DNA and RNA. Viruses range from 2–3 kilobase pairs (kbp) to about 1.2 megabase pairs (Mbp). A kilobase is a unit measuring a thousand base pairs; a megabase is a million base pairs, so a Mbp is a thousand fold larger than a kpb. But even a 1.2 Mbp virus is still an incredibly small entity requiring an electron microscope to see. As we might expect from this unit of measurement, our expert goes on to tell us that viruses contain either DNA or RNA, the stuff of life and why we tend to think of them as living things. In that same light, he tags on the fact that they possess a "genome protected by a virus-encoded protein coat called capsid." We'll return to that aspect later.

But now we have enough knowledge that we can begin to answer the question, 'What is a virus?' by confidently saying that *viruses are incredibly small snippets of genetic code*. In fact, much of this book will focus on the 'genetic code' part of this answer.

But knowing that still only partially answers the question. Our expert also twice describes viruses as 'parasites'—something whose own existence depends on some independent, living organism. Our expert clarifies this label by specifying that viruses are "obligate intracellular parasites," meaning that viruses, by necessity ('obligate'), within cells ('intracellular'), act like parasites. Now we might modify this notion by changing the noun 'parasite' to an adjective, 'parasitic,' because viruses aren't technically classified as parasites, which are larger, clearly alive, and share many similarities with other kinds of living things classified as *eukaryotes* (described below). But while viruses aren't classified as parasites they are clearly parasitic in that they depend on what a host provides to survive and replicate. As a descriptor, 'parasite' or 'parasitic'—with all the associated negative connotations—fits.

Our expert's description continues by answering the question of what a virus is by turning from its structure (small genetic code) and nature (parasitic) to further specifying its function, how it operates: as *an infectious agent*. Our expert informs us viruses survive by "directing the host cell machinery for the production of more viruses"—a function that is a benefit to the virus but a cost to the host—with the outcome that these more viruses "emerge from their respective host cell through lysis." In other words, *viruses are incredibly small snippets of genetic code that via infection hijack a host cell's genetic code to survive and multiply*. That multiplication is through the spread of the infection from cell-to-cell. *Lysis* is a particularly effective Greek term to name what happens in a replicated virus leaving a host cell to infect another; it means 'dissolution' of the host cell's membrane as a means of 'letting loose' the virus. It is a violent, destructive process.

Now we can return to our basic idea of a virus as an incredibly small snippet of *genetic material*. Remember, our expert told us a virus can either have DNA or RNA—but not both—for their viral genome. More on that in a moment. But let's round out our consideration of size here. As might be expected, DNA viruses are larger than RNA ones. In fact, this characteristic means DNA viruses are often called 'large viruses' and RNA ones termed 'small viruses.'

It is also worth noting that DNA viruses are more stable than RNA viruses; the latter have a higher mutation rate. The virus that causes COVID-19 is an RNA virus and, as most people are well aware, a major concern has been its mutations. That is because a treatment designed for one strain of a virus might not work as well (or worse, not at all) with a mutated strain.

We are interested in this book in the *genome* of a specific virus—the complete set of genetic information possessed by the virus causing COVID-19 (see chapter 3). Fortunately, viruses are small enough this means less genetic information and a simpler task to map and decipher than is true for most other entities. In fact, not all the information in the viral genome is of equal importance to us. So our task is simplified right from the start.

As we have seen already, the genetic information for any given virus is strung out in a measurable manner and specific units of measurement exist to describe various base pairs of DNA or RNA. Of course, knowing that tidbit won't be of any help unless we know what 'base pairs of DNA or RNA' means. This is our entry point into some basic genetics.

A Primer on DNA

We must begin by understanding some terminology belonging to the science of genetics. While most of us are fascinated by the subject a good many of us are also intimidated by it. Like other sciences, that of genetics uses a specialized vocabulary and so it can seem as if one is required to learn a foreign language—and that is besides trying to understand various concepts and biological processes. Taken together there can appear to be little incentive to tackle genetics. Yet to acquire an understanding of the virus that causes COVID-19 requires we must screw up our nerves and take the plunge. The good news is that we don't have to know everything to know enough to matter.

As we have been doing, our procedure will be not to shy away from scientific terminology but to break it down and explain it in more everyday English. We will be engaging in translating some basic science so that we facilitate our own comprehension and also can better communicate to others.

First of all, we likely already know that what we are depends on our DNA (deoxyribonucleic acid). Think of DNA as providing *instructions* that our body's cells will then carry out. These instructions cover all the matters needed for an organism to survive, develop, and eventually reproduce. Living organisms other than certain kinds of bacteria (eubacteria and archaebacteria) are known as 'eukaryotes' a Greek-derived compound ('eu'—'good' + 'kary-'—'kernel') descriptive of organisms having cells with 'good kernels,' i.e., a nucleus.

DNA Structure

Most of us learned in school somewhere along the way that the heart of the cell is the nucleus and the nucleus is where DNA is found. We doubtless somewhere along the way also were exposed to illustrations of DNA. Let's begin with one (like many in this book this one is from the National Human Genome Research Institute). It shows DNA's famous double helix structure. But we want to dive a little deeper into this structure to understand how DNA works.

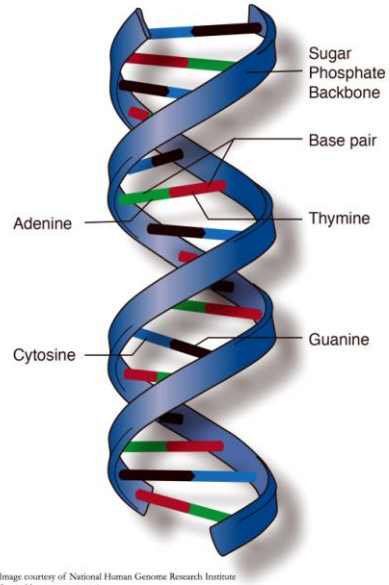
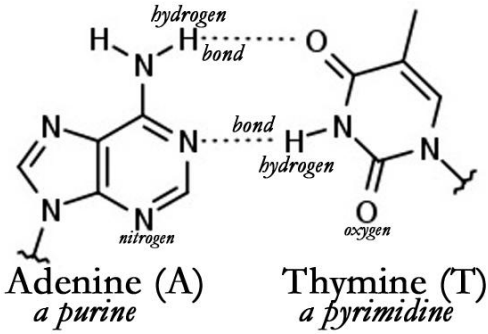


Image courtesy of National Human Genome Research Institute (https://www.genome.gov)

DNA's instructions are coded by four 'letters' (A, G, C, T)—the alphabet of genetics. This alphabet arranges into winding spirals of double strands attached to a backbone made of alternating sugar (deoxyribose) groups and phosphate groups. Each letter represents a *base*—a nitrogen rich organic molecule, of which there are two kinds: purines (A and G) and pyrimidines (C and T). These bases reliably pair as follows: A (adenine) bonds with T (thymine), and G (guanine) bonds with C (cytosine).

A *base pair* is thus two complementary molecules (A with T, or G with C) connected by hydrogen bonds. RNA (ribonucleic acid) alters the above picture slightly by replacing T (thymine) with U (uracil) and by being single stranded.

Why does this matter to us? Above, I described a virus as an incredibly small snippet of genetic code. Now we know that genetic 'code' uses the 'alphabet' of 'bases.' For those who took a class in chemistry the term 'base' may be familiar (a substance able to accept a hydrogen ion from another substance). Below is a close-up of the chemistry of one DNA *base pair*.



Note on each side of the hydrogen bond is a *base* (A to the left, T to the right).

Because this may be unfamiliar terrain for many of us, we may profit from supplementing the first image, which shows the traditional representation of a double strand of DNA, with another one.

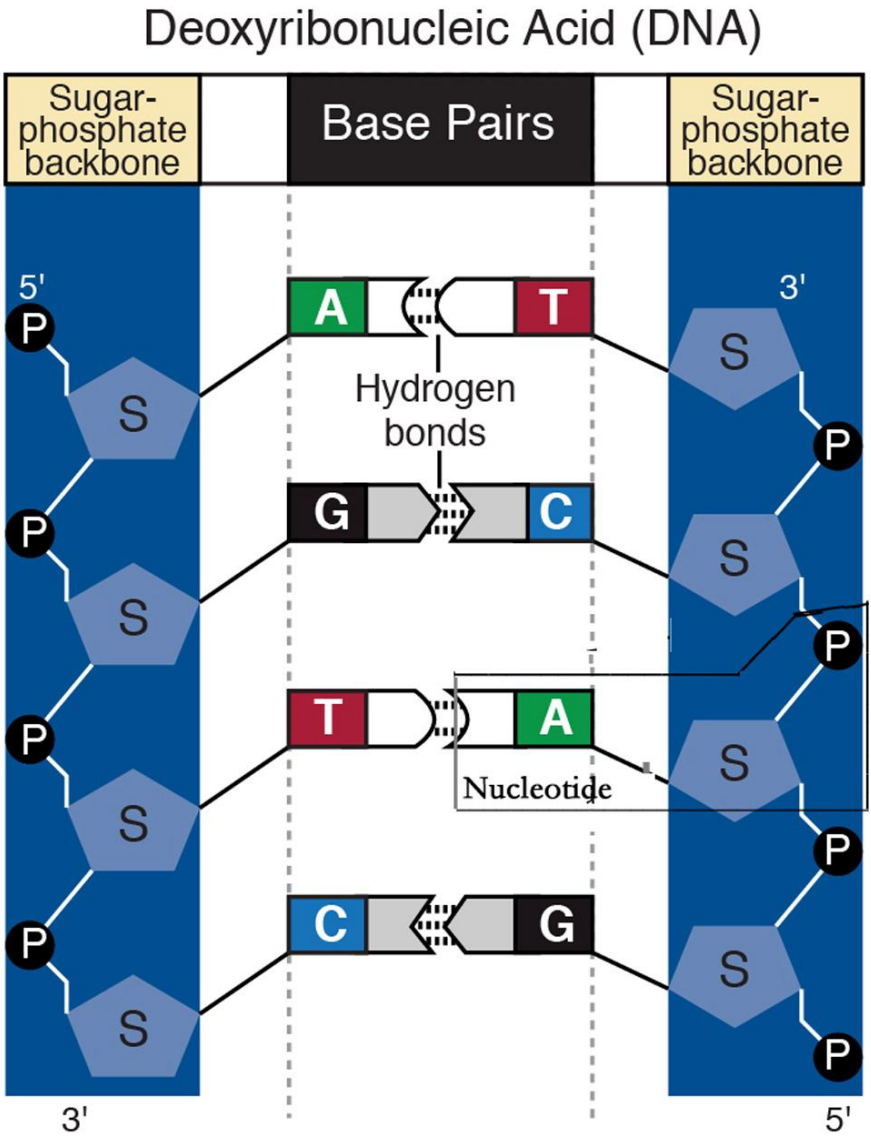


Image adapted from an illustration courtesy of National Human Genome Research Institute (https://www.genome.gov).

The above image conveys the same basic information but may make certain parts of it easier to grasp. For example, this structural representation probably makes it easier to see the hydrogen bonds between the two parts of a base pair

(e.g., A and T), while still seeing how each base pair connects to a sugar (S) phosphate (P) backbone.

The illustration just shown also uses another term we need to know: *nucleotide*—a structure formed of an organic base (A, G, T, C, or U) linked to a sugar molecule (S) linked to a phosphate (P). A nucleotide is a single chemical unit and as such is labeled a 'monomer.' When monomers are put together—a process termed 'polymerization'—the result is a 'polymer.' A *nucleic acid*—a large molecule ('macro-molecule') found in viruses and all cells—is a polymer. Both DNA and RNA are nucleic acids (i.e., chains of nucleotides), a kind of polymer.

The following illustration puts the previous two kinds of images together, which might further facilitate getting a sense of things. In this larger, more complete illustration the labeled bases (A, G, T, C) are again color coded which facilitates moving from the highlighted portion in the upper right hand of the drawing to the rest of it, which shows the complex winding staircase of DNA. Remember that the bases set side-by-side, connected with hydrogen bonds, are the base pairs. Our focus will be on base pairs.

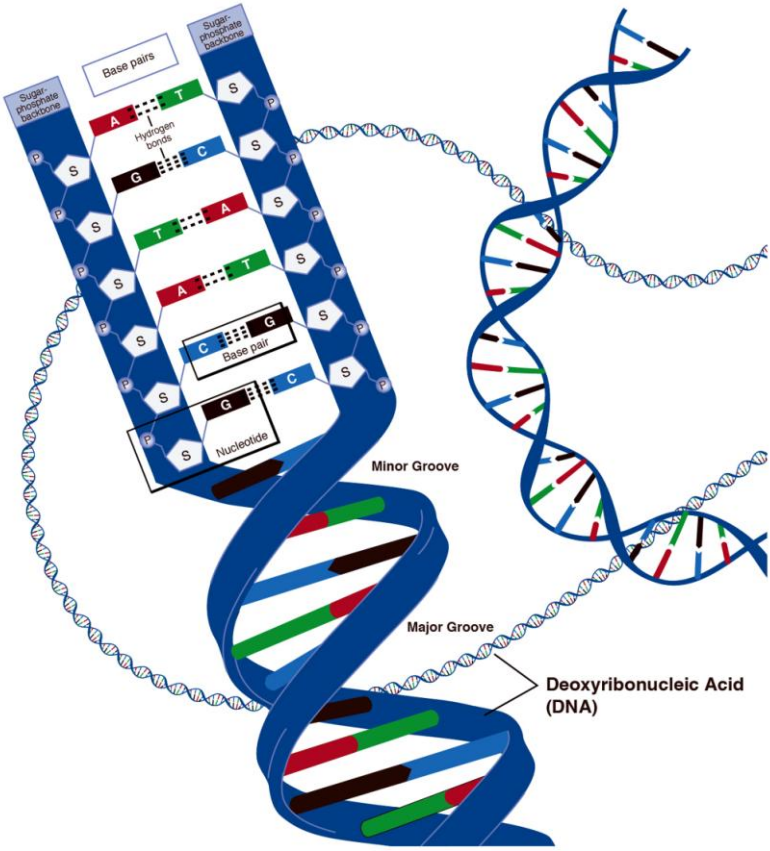


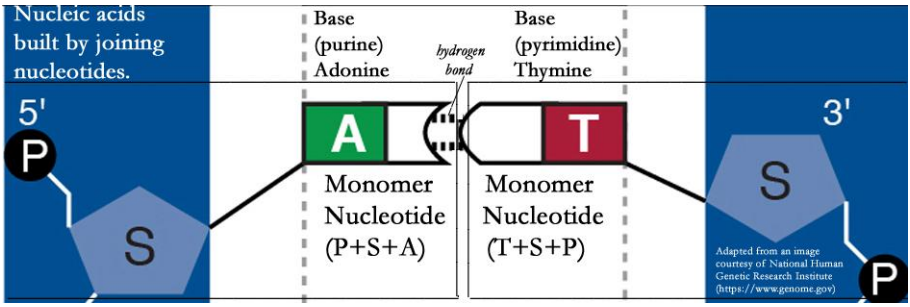
Image courtesy of National Human Genome Research Institute (https://www.genome.gov)

At the risk of appearing a bit tedious, let's conclude this portion by summarizing the vocabulary:

- DNA and RNA are *nucleic acids*,
- which are *poly*mers,
- which are formed by linking together *mono*mers called *nucleotides*.

Almost everyone is familiar with 'poly' ('many') and 'mono' ('one'), which makes remembering polymer and monomer easier. The '-mer' part of each word is Greek for 'part' or 'portion.' Polymer, then, means 'many parts,' and monomer means 'one part.' The 'nucle-' portion of both *nucle*ic and *nucle*otide refers to the nucleus of a cell, where DNA was discovered. The '-ide' in chemistry is used to identify simple compounds.

Put it all together visually and it looks like this:



Genes

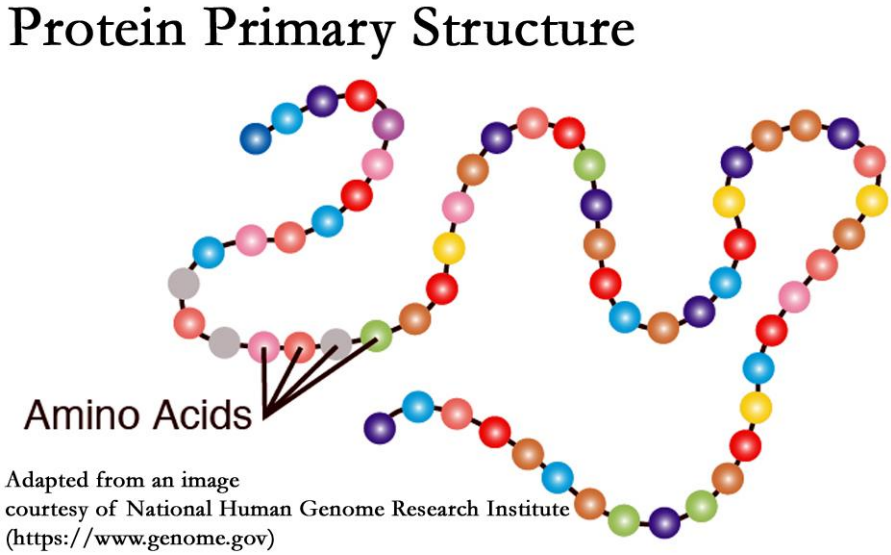
All this information about the components and structure of DNA may be interesting in itself, but we want even more to understand what it *does*. A nucleotide (a monomer) is a basic building block, and just like a child with toys, putting these building blocks together can make something—nucleic acid (a polymer). A DNA string, or sequence of building blocks, can make a *gene*—a set of specific instructions to make a protein. (But not all genes code for proteins and not all strings of DNA make genes.)

We are interested here in the protein-making genes. However, most of a sequence of DNA related to such genes—about 99%—goes to supporting the gene's instructions by regulating processes related to the protein (e.g., how it is made, when it is made, and how much of it is produced). This kind of DNA is called *noncoding DNA* to distinguish it from the coding DNA of genes.

Proteins

DNA and RNA, we have seen, are nucleic acids, which are polymers and a class of macromolecule (large molecule). A *protein* is another class of macromolecule. Proteins are even more complex than DNA, but unlike nucleic acids they are impermanent by nature. Proteins are polymers built in linear fashion

from strings of monomers known as amino acids. For any given protein—and there are many, many different kinds—the sequence of amino acids will correspond to a specific sequence of DNA that is the gene for that protein. A single protein molecule can be comprised of hundreds of amino acids.



Adapted from an image courtesy of National Human Genome Research Institute (https://www.genome.gov)

While we won't focus on amino acids, it is good to know these are organic compounds. The name is actually shorthand for the longer, formal name: α-amino carboxylic acid—a name descriptive of its chemical make-up, which is a basic amino group ($-NH_2$), plus an acidic carboxyl group (-COOH), plus an organic R group (a side chain). We saw earlier that the nucleic acid bases of DNA are nitrogen rich organic molecules.

Proteins are key actors in the interaction between viruses and human cells. The virus we are interested in has four main *structural proteins*—those that provide the virus its form, plus *nonstructural proteins* used in vital functions such as immune system evasion and viral replication, and *accessory proteins* that are less understood but assist viral work. So-called 'protein-protein interactions' (PPIs) are an integral aspect of viral infection. Because they lack ribosomes of their own, viruses are dependent on host cells' ribosomes. They hijack these ribosomes and use them to translate viral mRNA to synthesize viral proteins. Because this process is so critical to viral success it is important we understand how proteins are made.

In this very respect an important function of some proteins is acting as *enzymes*—biological catalysts, i.e., able to regulate a chemical reaction without themselves being permanently altered. Enzymes are crucial to cells' responsiveness to external stimuli. They also play critical roles in DNA replication, transcription, and repair. But our attention must be to the role of enzymes in protein production—one protein facilitating the making of another. That process

we shall examine in a moment. But first we must turn to DNA's partner, the nucleic acid designated RNA.

A Primer on RNA

Ribonucleic acid (RNA) is, like DNA, a nucleic acid. Compare the names:

- DNA: **d**eoxyribo | **n**ucleic | **a**cid
- RNA; **r**ibo | **n**ucleic | **a**cid

The difference in names highlights the sugar (S) component in each. In RNA the sugar is *ribose*; in DNA the sugar is *deoxyribose* (with one less hydroxyl group).

RNA nucleic acids differ from DNA in some notable respects. First, as said earlier, uracil (U) is only found in RNA, and thymine (T) only in DNA. Second, as a general rule, RNA molecules are smaller chains of nucleotides than are DNA molecules.

Viruses, we learned earlier, can be either DNA viruses or RNA viruses, and the specific one this volume is about is an RNA virus. That means RNA is important to understand. But we really can't understand it well apart from DNA.

Let's set it beside DNA to better capture the contrast.

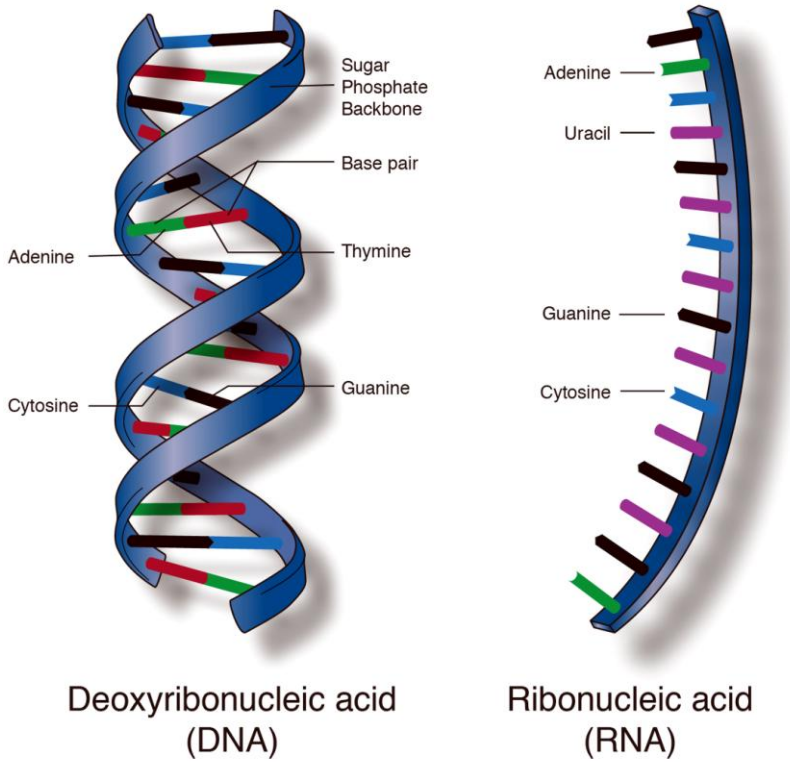


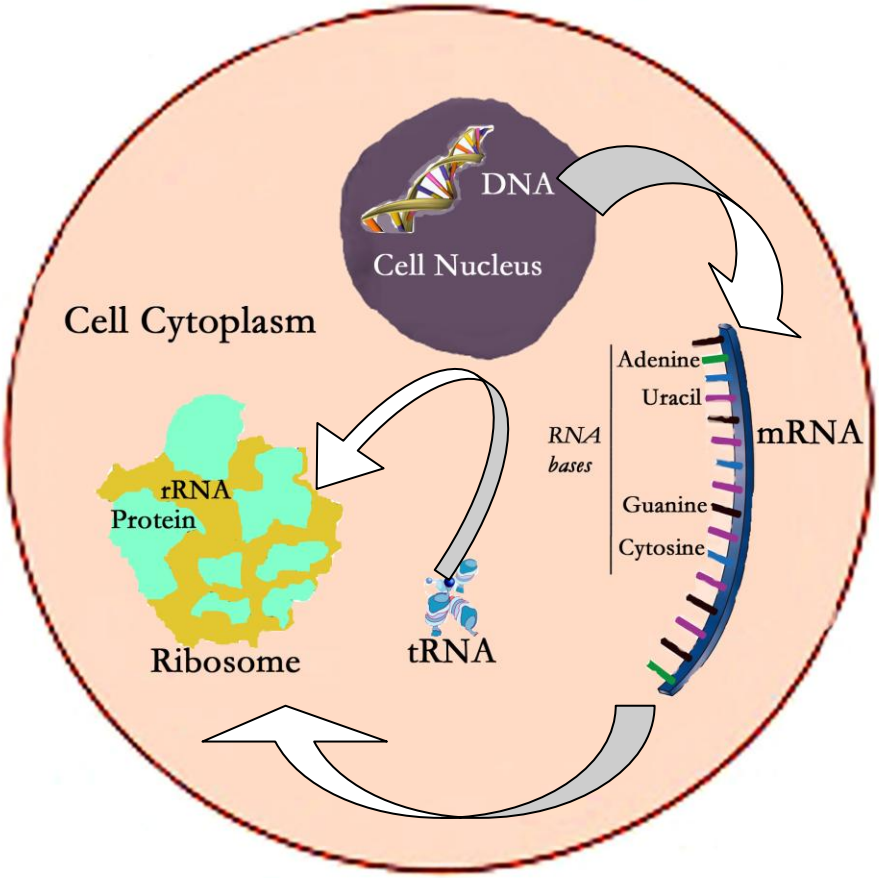
Image courtesy of National Human Genome Research Institute
(https://www.genome.gov)

One nice feature of this illustration is that it shows how RNA is *single-stranded* rather than double-stranded. However, it still has a backbone of sugar (S) and phosphate (P) groups. The four bases (A, G, C, U) attach to a sugar, just as in DNA. Remember, other differences between RNA and DNA are that RNA has uracil (U) as a base, but not thymine (T), and RNA is typically shorter.

RNA is traditionally differentiated into three types:

- mRNA—*messenger* RNA—as the name suggests, sends a message: it takes information from DNA to guide the making of proteins;
- rRNA—*ribosomal* RNA—is named for the ribosome, a kind of particle in cells, comprised of RNA and protein material; and,
- tRNA—*transfer* RNA—which, as the name indicates, carries to the ribosomes material needed for protein manufacture.

Although not to scale (which is also true of other illustrations), the following gives an idea of the relation of DNA to RNA and the three kinds of RNA.



Note that the shape of tRNA looks significantly different from mRNA, though both play important roles in the production of proteins. Messenger RNA (mRNA) provides the coding sequences derived from DNA for protein synthesis. Transfer RNA (tRNA) carries amino acids to the ribosomes as part of protein synthesis. While all three kinds of RNA are important, when discussing COVID-19 it is mNRA—*messenger* RNA—that takes center stage.

RNA and Protein Production: Transcription and Translation

We return now to a matter we started discussing before interrupting ourselves for an overview of RNA. We were about to examine how a protein functioning as an enzyme is involved in making another protein. This process involves both DNA and RNA.

The reason why both are involved entails remembering that DNA is locked in the cell nucleus. But its directions need to extend beyond the nucleus into the cell cytoplasm. It is there proteins are made. RNA provides the bridge.

Two major processes must occur for DNA to yield RNA for protein production. They are termed 'transcription' and 'translation.' The essential difference between these two processes is that transcription copies from one medium (DNA) to another (RNA)—but both are in the same 'language' (genetic code). Translation changes languages: RNA's genetic code (the original language) into the amino acid code (the other language), the language of a protein.

Transcription

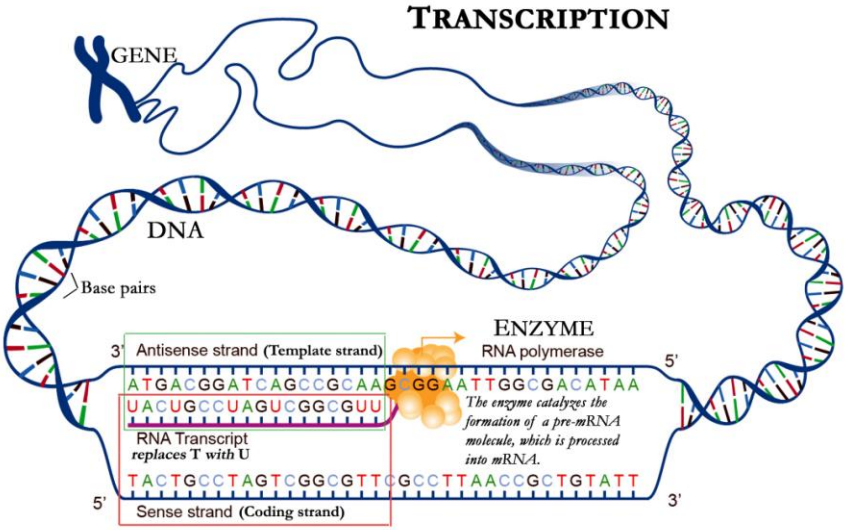
Making a protein first entails an enzyme 'reading' some DNA code (the alphabet of bases in base pairs), then 'rewriting' that code in a process termed *transcription*. Just like the word looks, it means to 'write' (the 'scribing' implicit in '-scription') 'across' ('trans-'), that is, copying the code *from* DNA *to* RNA—specifically mRNA.

Let's see if we can start with a simple explanation in basic English using a roughly analogous comparison. Somehow DNA in the nucleus of the cell needs to get its directions out into the body of the cell, where proteins are built. Like a scribe working with a monk living in a closet, an enzyme with a pretentious name (RNA polymerase) works with the DNA 'monk' trapped in the cell nucleus. The scribe carefully copies what the monk has written; the enzyme carefully copies what DNA has written. What has been copied, however, is now in a new medium; the tablet of the monk is not of exactly the same material as the tablet of the scribe. Similarly, the tablet of DNA is not exactly the same as the tablet of RNA. But this new tablet has the advantage that it can exit the 'closet' (nucleus) into the 'wider room' (cytoplasm), where it can be fruitfully used.

The process of transcription has three steps:

- *initiation*, in which a particular strand of DNA is identified.
- *elongation*, in which mRNA is built; and,
- *termination*, in which DNA signals the process should stop.

It looks like this:



Adapted from an image courtesy of National Human Genome Research Institute (https://www.genome.gov)

The above illustration shows how the DNA double strand near a gene unwinds into what is called a *transcription bubble* (the area at the bottom of the illustration showing the bases). In this bubble an enzyme (RNA polymerase) and transcription factors bind to the DNA strand known as the template, or antisense (or negative) strand. This is the strand that mRNA is built from; only one of DNA's strands is transcribed to synthesize mRNA. Transcription creates a RNA strand (the RNA Transcript) complementary to the DNA template strand (green box). This complementary RNA strand is almost identical to the DNA coding strand (the sense (or positive) strand of the DNA double strand) except that RNA's U bases have taken the place of DNA's T bases (red box).

The above is the short version. But let's unpack it.

Let's start in the middle of the bubble (where the orange is). Transcription uses an enzyme (RNA polymerase) plus other proteins in an assisting role ('transcription factors'). The label 'polymerase' might look somewhat familiar, and that becomes plainer if we break it apart: 'polymer' + 'ase.' We saw earlier that polymer simply means 'many parts.' The '-ase' is a language element used in naming enzymes, like a common last name for them. So 'RNA polymerase' is simply the descriptive name for a particular enzyme.

Now we can discuss the first step of transcription, called *initiation*, in which the stretch of DNA to be transcribed is identified. RNA polymerase, plus transcription factors, equals what is named the *transcription initiation complex*. The key term here is 'initiates'; the RNA polymerase and transcription factors bind to the DNA strand at a specific area, termed the *promoter region*. This region has three parts—core promoter, proximal promoter, and distal promoter—with the

core being where the RNA polymerase binding site is found. 'Proximal' means 'nearer' and 'distal' means 'further away' and both have reference to the core promoter. Both of these parts of the promoter region have transcription factor binding sites.

All of this can be illustrated as follows:

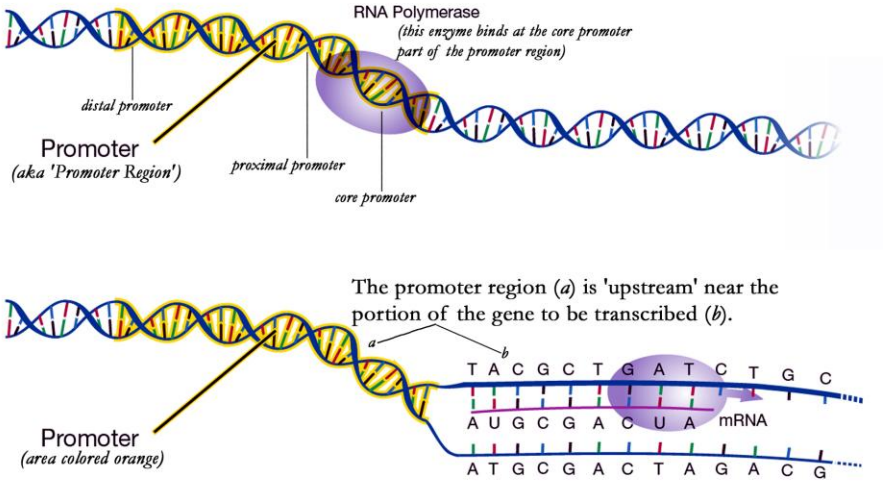
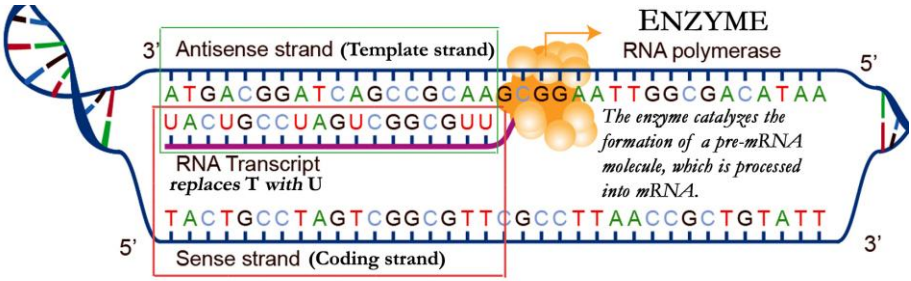


Image adapted from illustration courtesy of National Human Genome Research Institute (https://www.genome.gov)

Worth noting is that the promoter region commonly includes a specialized nucleotide sequence (TATAAA), referred to as the 'TATA Box.'

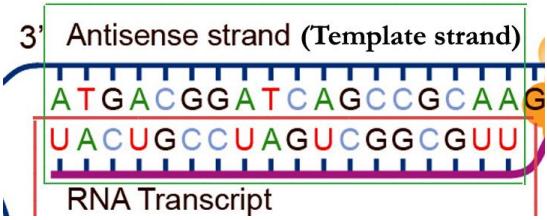
In the second step, *elongation*, a new mRNA strand is constructed as the RNA polymerase unwinds DNA and adds nucleotides. Here we need to focus on the boxes highlighted in an earlier illustration:



Adapted from an image courtesy of National Human Genome Research Institute (https://www.genome.gov)

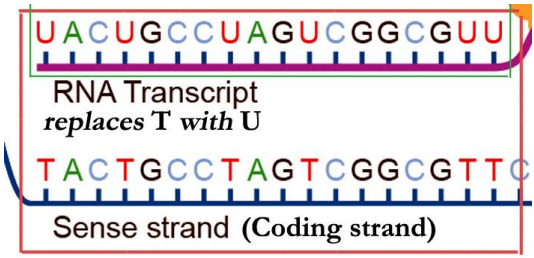
Notice first that DNA unwinds into two labeled strands. One of these is known as the Antisense, or Template strand (top of green box). The other is the Sense, or Coding strand (bottom of red box). We will focus on these by the names 'Template' and 'Coding.'

The step of elongation is named such because during it occurs the increasing in length of a new mRNA molecule (the RNA Transcript), based on the *template strand* of DNA (as shown in the green box, below).



The 'template' is a guide that controls transcription as the nucleotide sequence of mRNA is formed. Creating the RNA Transcript strand requires *complementary base pairing*. A 'complement' is something that when joined to something else makes a complete whole. We saw earlier that DNA has four bases and that they reliably pair in complementary fashion: A with T, and G with C (in both cases a purine (A, G) with a pyrimidine (C, T)). In that manner DNA has complementary base pairs. But RNA does not have thymine (T), so it must substitute uracil (U) for it in order to achieve a complementary base pair.

Now, look again at the red box shown overlapping the green box we just examined.



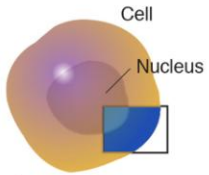
Here we can see that the RNA Transcript looks like the coding of the DNA coding strand, except that U has replaced T. The resulting RNA Transcript is complementary to the DNA template strand, just as the original sense strand of DNA was complementary to the antisense strand of DNA. In other words, the newly formed RNA strand is as complementary to the DNA template strand as was the original DNA sense/coding strand.

The final step of transcription is *termination*. A nucleic acid sequence in the gene being transcribed signals the elongation process to stop. As the mRNA has elongated it has also been peeling away from the template. RNA as a single-stranded molecule is very flexible and can fold into a variety of shapes—useful in protein building—but what we want to highlight here is that the mRNA chain does not remain hydrogen-bonded to the template strand. Separated from DNA the mRNA molecule can now exit the nucleus (and the DNA double helix reforms).

Transcription is the first major step in making a protein. Translation is the second major step—the actual 'making.' The mRNA molecule has been formed in the cell nucleus. It then departs the nucleus into the cell cytoplasm.

Here it directs the synthesis of a protein via a process known as *translation*. This is a form of transfer of information; the information of DNA is 'carried over' (translated) from nucleus to cytoplasm, and 'expressed in another language' (translation). The DNA message first transcribed by an enzyme into mRNA in the cell nucleus is now translated in the ribosomes of the cell cytoplasm in both the senses described above.

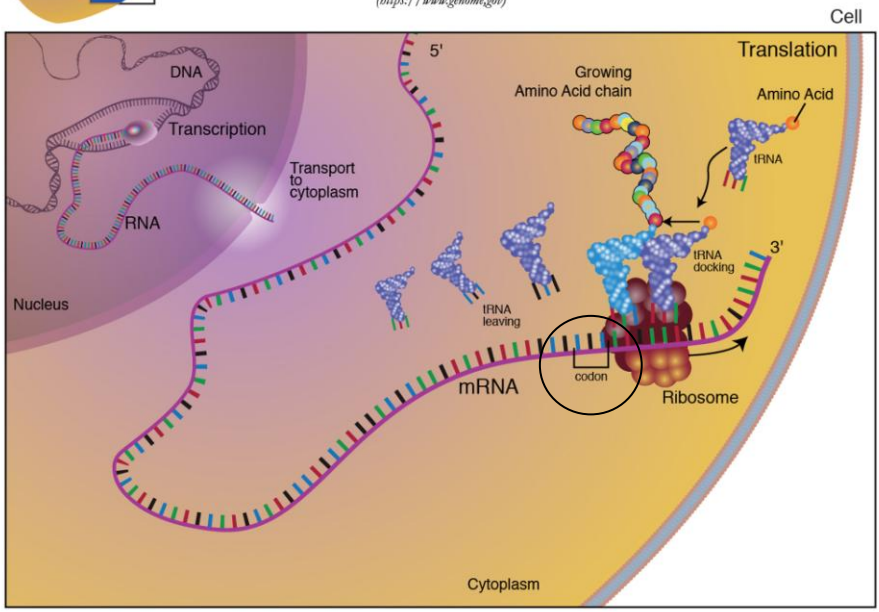
Translation is exactly that: the mRNA is 'read' and related to the amino acid sequence in a manner analogous to translating the vocabulary of one language into that of another. The language of RNA is 'genetic code'; the language of a protein is 'amino acids.' What happens in the ribosome translates the genetic code language into the amino acid language—like a language translator renders Greek into English. In language translation, to make the original language make sense in the new one the vocabulary of the new language has to be put into sensible order. In genetic translation mRNA instructions direct, in order, the assembly of the amino acid chain that makes a protein.



TRANSLATION

Transcription has occured in the cell nucleus (shaded area, upper left of the expanded inset, below).
Translation transpires in the cell cytoplasm.

Adapted from an illustration courtesy of National Human Genome Research Institute (https://www.genome.gov)



In the preceding illustration we see mRNA at the ribosome being translated. Let's start with the portion I've circled—the *codon*. The word derives from Latin *codex*, 'a book,' which in Old French became *code*, 'a law-book,' or 'a system of laws.' The basic sense is carried over into biology where 'codon' ('code' + suffix 'on') refers to a meaningful system of arranged nucleotides that determine which amino acid goes where. As can be seen, a codon is *a sequence of three nucleotides*. But it isn't just a random sequence; each codon of mRNA corresponds to a specific amino acid (a 3 bases to 1 amino acid ratio).

CODONS

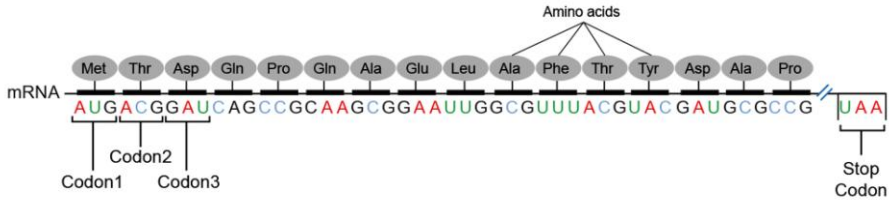


Image courtesy of National Human Genome Research Institute
(https://www.genome.gov)

There are 61 different codons referring to 20 specific amino acids, one of which (AUG) is reliably at the start, and 3 other codons (UAA, UAG, UGA) used only as 'stop!' signals to tell the ribosome when to quit translating. As may be obvious by now, *the codon is the deciphering key* that allows one language (genetic code) to be translated into another (amino acid code).

Look again at the last paragraph: 61 different codons correspond to 20 specific amino acids. That means the system is *redundant*—more than one codon can code a given single amino acid. Most amino acids are encoded by more than one codon (2–6 each for various of 18 amino acids), and these codons are *synonymous codons*. However, not all synonyms are equally preferred; certain codons are preferred and this reality is termed *codon usage bias* (CUB).

The importance of this matter for us rests in three important implications. First, different synonymous codons at a specific place in an mRNA sequence may result in different effects on mRNA structure and stability. Second, synonymous codons can influence gene expression. Finally, codon usage bias has been observed in the genomes of all organisms—including viruses.[3]

CUB has been variously explained. One model finds the cause in mutation pressure, another finds it in translational selection. In most viruses, rather counterintuitively, the codon usage of the viral genome shows codon usage patterns different from the codon usage patterns of their hosts. This means that how some viral genes with different codon usage patterns are expressed efficiently remains somewhat a mystery.[4] In RNA viruses, like the one we are concerned about, CUB is generally low to moderate.[5]

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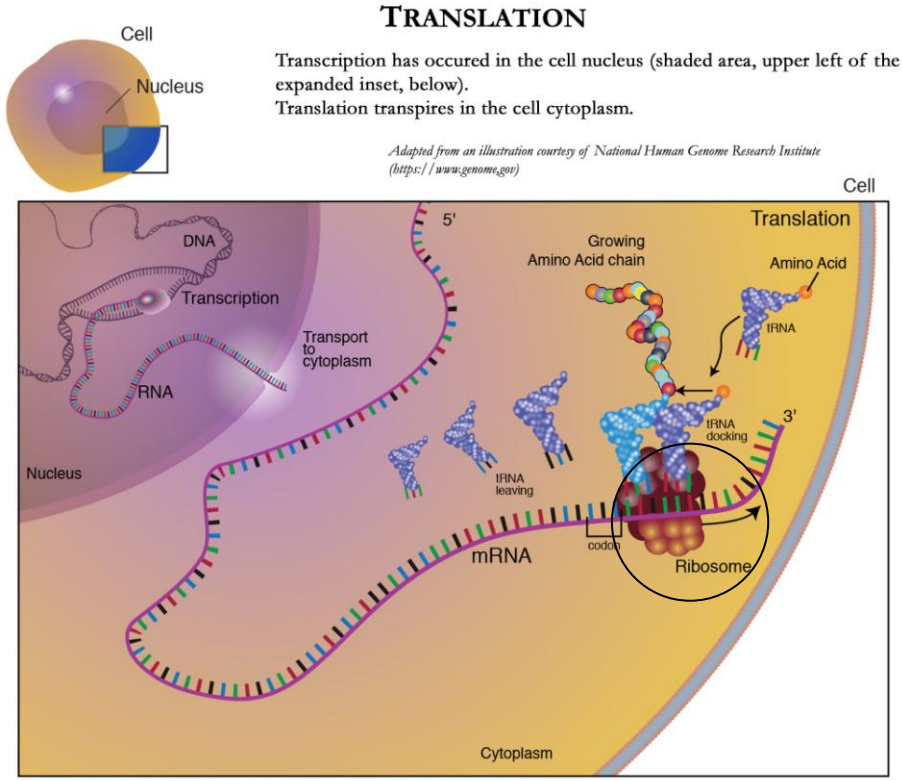
[3] Mitra, Ray, and Banerjee, "Synonymous Codons Influencing Gene Expression in Organisms."
[4] Jitobaum et al., "Codon Usage Similarity."
[5] Belalov and Lukashev, "Causes and Implications of Codon Usage Bias," 2.

Like transcription, translation entails three distinct steps and these are often provided the same names as we saw for transcription:

- *initiation*, in which binding of a ribosome to mRNA occurs;
- *elongation*, in which assembled amino acids are joined together; and
- *termination*, in which the ribosome reaches a stop codon.

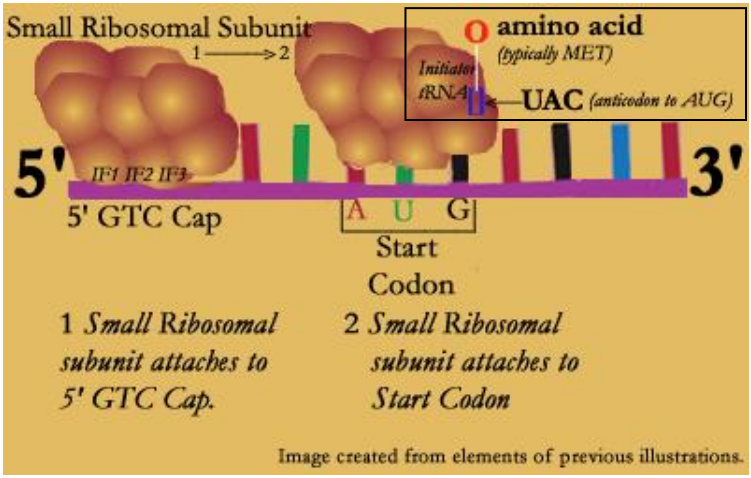
Now let's repeat our illustration for translation and examine initiation.



Initiation of translation features the ribosome, the cell cytoplasm's translation center (the circled part above). At the ribosome all three kinds of RNA are involved. Let's first look at *ribosomal* RNA (rRNA). This is the kind of RNA that binds with proteins to form ribosomes. Every ribosome has at least one large molecule of rRNA and one small molecule of rRNA; when these join with proteins they form a rather complex structure comprised of two subunits not so imaginatively named 'large subunit' (designated 50S) and 'small subunit' (30S).[6] The small subunit binds to the mRNA sequence using a tRNA sequence known as an 'initiator.' Together, the mRNA, ribosome, and initiator with an amino acid attached form a translation *initiation complex*.

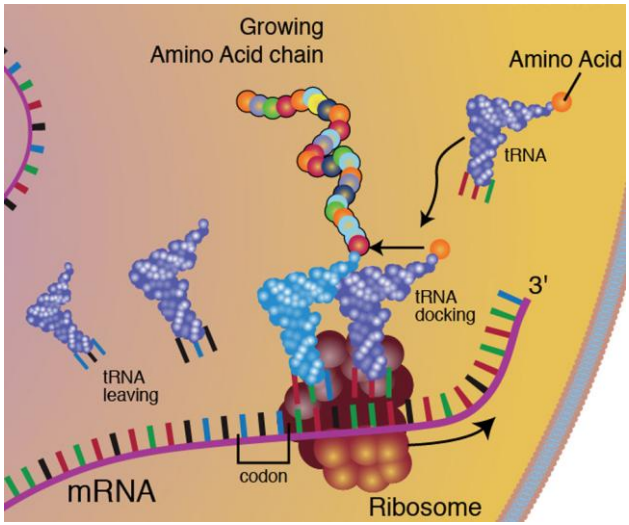
[6] The 'S' stands for 'Svedberg unit' which indirectly permits a measure of size based on sedimentation velocity.

The small unit of the ribosome recognizes a *GTP* cap—a protective end structure on mRNA made of Guanosine-5'-triphosphate (GTP) tubulins. This cap, added while processing was occurring in the nucleus, when identified by the ribosome is attached to using three initiation factor proteins (IF1, IF2, IF3), as shown at the left side of the illustration below.



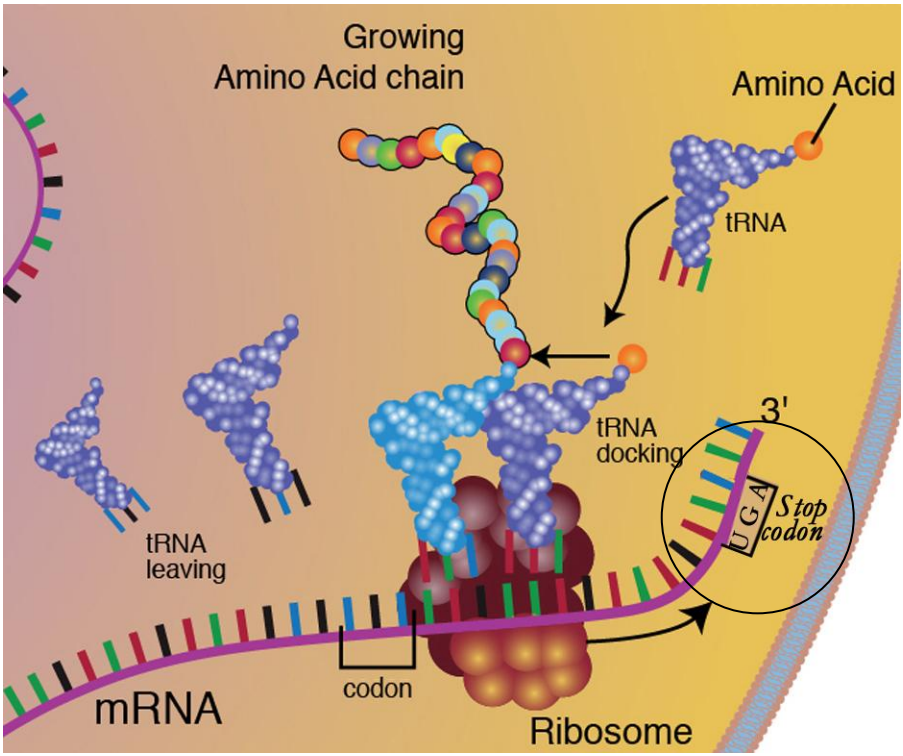
As shown on the right side of the above illustration, the ribosome then moves 'downstream' to the translation starting point proper, always at a codon named the 'start codon' (typically AUG). As highlighted in the box at the upper right of the illustration, *initiator tRNA*—like all transfer RNA—acts like an adaptor, with one end attached to an amino acid (typically methionine (MET)), and the other end to an anticodon (i.e., complement) to the codon AUG. This process of binding to mRNA, beginning at the start codon, is called 'docking.'

Let's return to our base illustration and refocus our attention:



This illustration highlights a second major step in translation—*elongation*, and tRNA is critical. Like a ratchet grinding along in one direction (shown by the arrow at the ribosome), the assembled amino acids are joined together as the ribosome moves along the mRNA. But read the illustration from right to left: as the ribosome moves inexorably along, for brief periods as each new codon is reached there are two tRNA molecules on the mRNA sequence. When the amino acid carried by a tRNA molecule is bound to the codon of mRNA the tRNA has completed its transfer and is released (shown in blue and labeled tRNA leaving). The amino acids in this chain are linked by what is called a *peptide bond* (or *amide bond*), a chemical process we need not expand on here.

We need only a minor change to reach our last step in translation.



The final step—*termination*—is simply described: when the ribosome reaches a stop codon it does as instructed. The circle highlights a stop codon—in this illustration UGA (but UAA and UAG are other stop codons).

Most simply put, beginning where rRNA docks at the start codon the ribosome moves along the mRNA with the tRNA molecules linking amino acids with their corresponding mRNA codons, processing them into a protein chain until a stop codon signals the machinery to stop. At the end of this process a protein has been synthesized. Once created the protein then performs the work for which it has been created.

Is a Virus a Living Organism?

We now have enough of a sense of basic genetics to better understand what we will say later about the virus that causes COVID-19. But to tie things together, let us return to a basic issue. We began this chapter by observing that the question whether viruses are living organisms remains an open one. We naturally think of genetic code as the language of life, so if a virus is so constituted it is easy to conclude it is alive. Yet a moment's reflection should remind us that 'incredibly small snippets of genetic code' does not necessarily signal life any more than relatively small snippets of letters must form words, let alone books.

To go deeper into the issue we have to explore the boundary between chemistry and biology. All biological forms use chemistry, but not all chemistry is biological in nature. Where the two meet we call it *organic chemistry*.

'Life' is commonly understood as including certain abilities, including the ability to reproduce. We can focus on this idea because it best illustrates the problem in understanding what a virus is. Viruses are usually said to 'replicate' rather than to 'reproduce,' and that is how we have described them.

Now the ability to replicate can be viewed as a form of reproduction—so we can argue that a virus meets that important quality of being 'alive.' However, as we have seen, *viruses are unable to replicate on their own*. They are incapable of independent replication. Instead, they depend on metabolic processes possessed by some unambiguously alive other organism. To replicate they must invade a host and hijack some of its abilities.

We noted earlier that this description likely brings to mind *parasites*—organisms that live on or inside other organisms. Ready examples are found in *bacteria*, very small, single-cell organisms. But bacteria are unlike viruses in a number of ways, including that they do not depend on their host to reproduce.

Another difference from bacteria, and important to us here, is that bacterial diseases are commonly susceptible to treatment by antibiotics, but viruses are not. So when a virus such as the one that causes COVID-19 occurs, antibiotic remedies to cure it are not readily available. The matter of anti-*viral* treatments we shall take up later on.

From a psychological perspective it may be difficult for us to not personalize a threatening virus. After all, we are a species that gives human names to hurricanes and often treats forces of Nature as though possessing human personality, will, and intent. I prefer to think of viruses as nonliving snippets of genetic code—sometimes malicious and, like a computer virus, unwelcome and destructive. Such code should ideally be prevented from infecting the host.

We must go deeper. Near the beginning of this chapter we discovered that more than 4,000 viruses have been identified. They have been classified into some 71 'families' (another personification). The idea of *viral families* permits us to narrow the scope of our study. It is to one such family of viruses we must now turn.

Chapter 2
Coronaviruses

The 'family' to which the virus causing COVID-19 belongs is named *coronaviridae* (popularly called *coronaviruses*). Like most scientific names, this one is descriptive. The most distinctive feature of a coronavirus when observed under an electron microscope (see image below) is an extruding feature (a 'spike') that ends in a 'crown' (Latin *corona*).

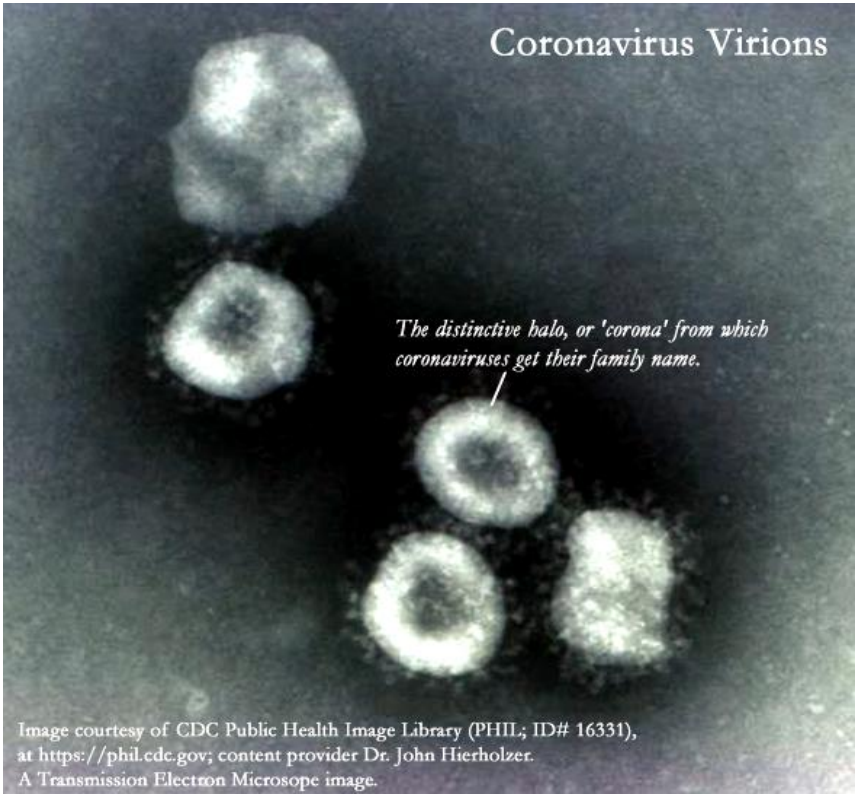


Image courtesy of CDC Public Health Image Library (PHIL; ID# 16331), at https://phil.cdc.gov; content provider Dr. John Hierholzer. A Transmission Electron Microsope image.

The above 1974 image was extracted from a purified culture. The label *virions* is one derived from 'virus' plus the subatomic particle suffix '-on.' Virions display the complete virus form outside a host cell, ready to infect it. That form includes the RNA core and its protein coat ('capsid'). The distinctive spikes that cast a halo in the picture—the 'corona'—are not merely what give the family name to this kind of virus, but they are also what have drawn the most attention. That is not because they are so distinctive, but because of the role they play and because of their vulnerability. In other words, the very feature that makes a coronavirus so distinctive and plays such an important part in how they function is also in a crucial way its Achilles' heel.

The most distinctive feature of a coronavirus is even clearer in the following illustration. Virtually everyone by now has seen one or another artistic representation of a coronavirus, and so this should look familiar.

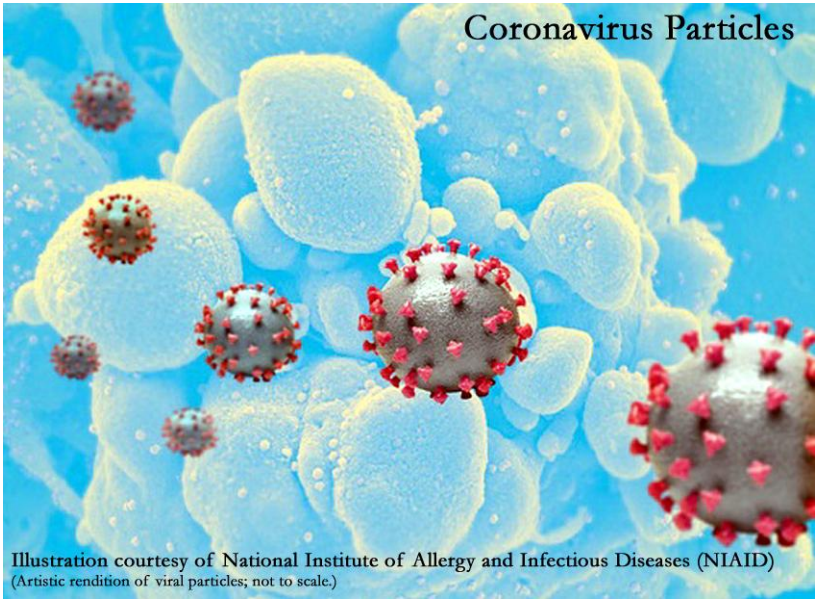


Illustration courtesy of National Institute of Allergy and Infectious Diseases (NIAID)
(Artistic rendition of viral particles; not to scale.)

The advantage of this kind of image lies in its showing more clearly the very striking form of the coronavirus spike.

Naturally, having seen what a coronavirus looks like we will be interested in learning more about these fascinating spikes as well as the remainder of the structure of these viruses. But before we get into such details it is desirable to look at a little family history.

Anti-vaxxers have sown doubt about the safety and efficacy of the COVID-19 vaccines based on how rapidly they were developed. But part of an effective response to this claim is grasping how much study had gone into coronaviruses *before* COVID-19 reared its ugly head. Because researchers already possessed an extensive history with this virus family, many of its family characteristics and habits were already known before vaccines were beginning to be developed.

In this way vaccine developers were not starting from a resting place; they already had a running start on the virus. It is true that COVID-19 burst dramatically onto the scene, and it is also true that the world was not as ready for it as it might have been—and *should* have been. But it is not true that scientists were caught completely flat-footed. In fact, because of what had been done in previous study of the coronavirus family—work extending back now more than *90 years*—vaccine developers had a solid science foundation to begin from.

Knowledge is the best preparation; baseless beliefs flowing from ignorance (or, worse, selfish interests) only arouse and perpetuate fear. So enough preaching from me and let's look at this history of study of coronaviruses.

A Brief Family History

Some highlights of the study of coronaviruses can be arranged as follows.[7]

(1924–25)	Retrospective identification of infectious bronchitis virus (IBV)
1931	First identification of a coronavirus, first named IBV
1937	Isolation of IBV and first cultivation of the virus
1949	Isolation of murine hepatitis virus (MHV) in mice
1966	Discovery of first human coronavirus (HCoV-229E)
1967	Discovery of another human coronavirus (HCoV-OC43)
1980	First international coronavirus conference (Wurzburg, Germany)
1986	Polyprotein processing of coronavirus
1987	First sequencing of a coronavirus genome (IBV)
1992	First recombinant coronaviruses engineered by targeted recombination
1995	Discontinuous transcription
2000	Full-length reverse genetic clone
2002	Electron microscopy of double-membrane vesicles
2002–03	SARS-CoV identified in first serious human coronavirus epidemic
2004	Discovery of another human coronavirus (HCoV-NL63)
2005	Discovery of another human coronavirus (HCoV-HKU1)
2008	Electron tomography of double-membrane vesicles
2011–15	Five year study finds 11 newly discovered SARS-CoV strains
2012	MERS identified in second serious human coronavirus epidemic
2019	SARS-CoV-2 identified as causal agent of COVID-19
2020–22	COVID-19 pandemic

Coronaviruses have been known since the start of the 1930s. In 1931, an infectious respiratory disease spread among chickens in the United States. The outbreak was first noted in North Dakota and provided the name 'Infectious Bronchitis Virus' (IBV) in a 1937 report,[8] the same year it was isolated and cultivated.[9] Subsequently it was identified as the causal agent of an earlier outbreak among chickens in 1924–1925 in the Upper Midwest and in New England.[10] Although still known by its original name, IBV is a coronavirus. Like its cousin that causes COVID-19, it is highly infectious—up to a 100% morbidity rate in a flock—and very deadly: anywhere from 25–30%, up to 80%, or higher. Also like COVID-19, IBV has been discovered to affect more than the upper respiratory tract; the virus has been found in other organs, including the kidneys. Also like its cousin, it has been found not only in the United States, but Europe, Africa, the Near East, and China.[11]

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[7] For timelines and brief remarks, see V'koski et al., "Coronavirus Biology and Replication," 157 [Box 1]; also Helmy et al., "The COVID-19 Pandemic," 3; also see Kahn and McIntosh, "History and Recent Advances in Coronavirus Discovery"; alse see Weiss, "Forty Years with Coronaviruses."

[8] Schalk and Hawin, "An Apparently New Respiratory Disease." Also see Fabricant, "The Early History of Infectious Bronchitis."

[9] Beaudette and Hudson, "Cultivation of the Virus of Infectious Bronchitis."

[10] "Coronaviradea," 444.

[11] Awad et al., "An Overview of Infectious Bronchitis Virus in Chickens."

As the timeline shows, early identification of what would come to be known as coronaviruses was in the animal world. First it was IBV in chickens, but by the end of the 1940s it was also murine hepatitis virus (MHV) in mice. In the 1949 study isolating the virus, MHV-infected mice showed illness at the end of a 24 hour period and often died within 36 hours.[12] Although a disease afflicting mice, MHV has had relevance to human diseases, often being studied in order to better understand hepatitis in humans as well as multiple sclerosis.[13]

The actual naming of these viruses as 'coronaviruses' came in the late 1960s, after the discovery of the first coronaviruses to infect humans (HCoV-229E and HCoV-OC43). In 1968, an informal group of virologists sent the conclusions of their research to the journal *Nature*. In the 'News and Views' section of the distinguished publication it was noted that the name was suggested by the observed fringe of rounded projections resembling the solar corona—a feature seen in a variety of viruses. This suggestion was approved by a study group under the International Committee for the Nomenclature of Viruses (ICNV).[14] The name has subsequently became widely adopted.

The 1980s saw the first international conference on coronaviruses. In the late 1980s was successful study of the polyprotein processing of coronaviruses. This was critical because *polyprotein processing* is the making of many functional proteins from a single open reading frame (ORF)—a key process for viruses. About the same time was the first sequencing of a complete genome (IBV).[15]

The emergence of "Severe Acute Respiratory Syndrome Coronavirus" (SARS-CoV), and the epidemic it spawned at the start of the 21st century, drew sharp attention and some urgency because of its high fatality rate (10%). In 2002, because of this epidemic, SARS-CoV's genome was decoded.

The new diligence inspired by SARS led to identification of two more human coronaviruses (HCoV-NL63 and HCoV-NL63) in the years immediately following the epidemic. It also led to such research efforts as a five year study of SARS related coronaviruses in a cave in Yunnan Province, China, inhabited by multiple species of horseshoe bats. That one study discovered eleven new coronaviruses and determined their full-length genomes.[16]

When MERS-CoV emerged in 2012, with an even more dramatic mortality rate than SARS had—more than 1-in-3 (36%)—the coronavirus family became even more a matter of serious worldwide scientific scrutiny.[17] The situation was then exacerbated by the COVID-19 pandemic. But, because of decades of study by dedicated researchers when the pandemic struck at the end of 2019 a wealth of information was already at hand to draw upon.

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[12] Cheever et al., "A Murine Virus."
[13] Körner et al., "Of Mice and Men," 3.
[14] "Virology: Coronaviruses," 650.
[15] Weiss, "Forty Years with Coronaviruses." Subsequently the complete genomes of HCoV- 229E and HCoV-OC43 were sequenced as have been the more recent coronaviruses.
[16] Hu et al., "Discovery of a Rich Gene Pool."
[17] See V'kovski et al., "Coronavirus Biology," 157, Box 1.

A highlight of the history of study of coronaviruses has been the sequencing of complete genomes. The first, as noted above, was HBV. But because most known coronaviruses affect animals rather than people, and the effects of the human coronaviruses known in the 20th century were mild, there was little incentive or urgency for genetic sequencing. The SARS epidemic (2002–03) changed that. Before SARS there had been fewer than ten coronaviruses whose complete genomes had been sequenced (including the two human coronaviruses then known: HCoV- 229E and HCoV-OC43). But after SARS, between 2003 and April, 2010, fifteen more coronaviruses were discovered and their complete genomes sequenced, including other human coronaviruses.[18] By May, 2021, more than 1.2 million coronavirus genomes from 172 countries and territories had been gathered and shared on an online data platform—the Global Initiative on Sharing Avian Influenza Data (GISAID).[19] By the middle of November, 2021, the number of genome sequence submissions for the coronavirus causing COVID-19 numbered 5,209,469.[20]

The subsequent burgeoning of information has produced important knowledge that could be drawn upon when the COVID-19 pandemic erupted. We will profit from brief consideration of a few items. They include:

- Coronaviruses have a *relatively large genome* (for a virus).
- Coronaviruses, though relatively stable, are *liable to mutations.*
- Coronaviruses share a *conserved order of seven genes*.

A Relatively Large Genome

First, coronaviruses possess the largest genomes of any known RNA viruses (26.4–31.7 kilobases (kb)—a kilobase measuring a thousand base pairs). The importance of this fact rests in the adaptive edge it provides. A larger genome means greater *plasticity*—the ability to adapt to different environments and environmental changes. As we might anticipate, this means an enhanced ability to modify genes.[21]

Liable to Mutations

Second, as virologist Malik Peiris points out, "Genetic recombination readily occurs between members of the same and of different coronavirus groups providing opportunity for increased genetic diversity."[22] This diversity facilitates mutations advantageous to the virus but disadvantageous to host immune systems trying to ward off infection. The mutation rate of any virus is a significant factor in such matters as cross-species passage and the risk of infectious disease

[18] Woo et al., "Coronavirus Genomics and Bionformatics Analysis," 1805.
[19] Maxmen, "Popular Genome Site."
[20] As of Nov. 17, 2021. Accessed online at https://www.gisaid.org/.
[21] Woo et al., "Coronavirus Genomics and Bionformatics Analysis," 1806.
[22] Peiris, "Coronaviruses," 587.

outbreaks. RNA viruses, as a general rule, have higher mutation rates than DNA viruses.[23] It should be noted that a great many mutations end up having no demonstrable significance. This matter shall be explored more later.

A Conserved Order of Seven Genes

Third, all of the coronavirus family members have in their genome a *conserved order* of seven genes. This means all of them share an order of sequence for these RNA nucleic acids, and they are in an order of direction (5′ to 3′).[24]

5′	ORF1a	ORF1b	S	ORF3	E	M	N	**3′**

ORF abbreviates *Open Reading Frame*—here referring to a portion of RNA without a stop codon (thus, 'open'), that can be read by a ribosome to make a protein (thus, 'reading'), and together constituting a frame of reference ('frame'), which is really just the genetic sequence between any given start and stop codons. There are three genes characterized as ORF (1a, 1b, and 3).

Two open reading frames—ORF1a and ORF1b—are collectively referred to as ORF 1ab; this occupies about two-thirds of a typical coronavirus genome. In the various subgroups of the coronavirus family there are variable numbers of additional ORFs. Most of these are preceded by a *transcription regulatory sequence* (TRS)—a nucleic acid sequence that can increase or decrease transcription. This mechanism of regulation has been considered to be important in bringing about greater similarity in RNA recombination (i.e., 'a high frequency of homologous RNA recombination').[25] Study of ORFs has been important in tracing the evolution of a coronavirus; for example, research early in the pandemic suggested genes associated with ORFS 1ab, 3a, and 8 driving early evolution of the coronavirus behind COVID-19.[26]

The structural and accessory proteins make up about one-third (10 kb) of the viral genome. The accessory proteins appear to be nonessential for replication but significant in the development of disease. One way they do this, in some instances, is by inhibiting the innate immune response.[27]

Four genes code for specific proteins: Spike (S), Envelope (E), Membrane (M), and Nucleocapsid (N). All of these coronavirus genes occur in the 5' to 3' order in the same way as shown above.[28] Coronaviruses also exhibit certain regularities in these major structural proteins. These matters, though, will be treated in the following sections where we focus on these four proteins.

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[23] Sanjuán et al., "Viral Mutation Rates."
[24] See, among many others, Wang, Grunewald, and Perlman, "Coronaviruses"; also Woo et al., "Coronavirus Genomics and Bionformatics Analysis."
[25] Woo et al., "Coronavirus Genomics and Bionformatics Analysis," 1806.
[26] Velasquez-Salinas et al., "Positive Selection of ORF1ab, ORF3a, and ORF8 Genes."
[27] Wang, Grunewald, and Perlman, "Coronaviruses," 2. Also see Liu, Liang, and Fung, "Human Coronaviruses-229E, -OC43, -NL63, and HKU1," 431.
[28] Brian and Baric, "Coronavirus Genome Structure and Replication."

Family Characteristics (Structural and Functional)

The 'corona' that gives coronaviruses their name is hardly the only feature we must note with this family. Let us begin by listing a few things to remember. Coronaviruses are:

- RNA viruses (i.e., small compared to DNA viruses);
- variant in size and shape (i.e., *pleomorphic*);
- possessed of a protein shell (i.e., *capsid*);
- surrounded by an outer wrapping (i.e., a *lipid membrane envelope*); and,
- distinguished by spikes on the viral surface (i.e., *peplomer*).[29]

Each of these merits some detail as we work from the inside outward.

RNA Viruses

All coronaviruses are RNA viruses. As we saw in chapter 1, RNA is a single-stranded string of genetic code (unlike DNA's double-stranded structure). Viruses can be either 'large' (DNA viruses), or 'small' (RNA viruses). All coronaviruses are *very* small. Virologist and evolutionary biologist Edward Holmes links the smallness of RNA viruses to two other important characteristics: they are highly prone to mutation and they have very high numbers of descendants.[30] We may add two others: all the major viral killers of human beings are RNA viruses, and they are harder to extinguish than DNA viruses.[31]

Variant in Size and Shape (*Pleomorphic*)

Although small viruses, within their own viral order coronaviruses are relatively larger in size—but still quite variable. This variability coupled with such extreme small size it requires using an electron microscope to see them has resulted in various estimates of just exactly how small they are. Before the COVID-19 pandemic they were reported in different sources as varying between 60–220 nanometers (nm) in diameter (2012),[32] or between 80–220 nm (2017);[33] with the identification of the new coronavirus, that range has been expanded to 40–220 nm (2021).[34] To put this into more graspable terms, a nanometer represents *one billionth of a meter* (1 meter=39.37 inches). A period written at the end of a sentence by a pencil is about 1.6 millimeters (mm), the equivalent of 1,600,000 nanometers, or about 7,273 times larger than the largest coronavirus.

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[29] Peiris, "Coronaviruses," 588.

[30] Holmes, *The Evolution and Emergence of RNA Viruses*, 6. In fact, Holmes argues (p. 7), "the key patterns and processes of RNA virus evolution are in large part a function of their intrinsically high rates of mutation. . . ."

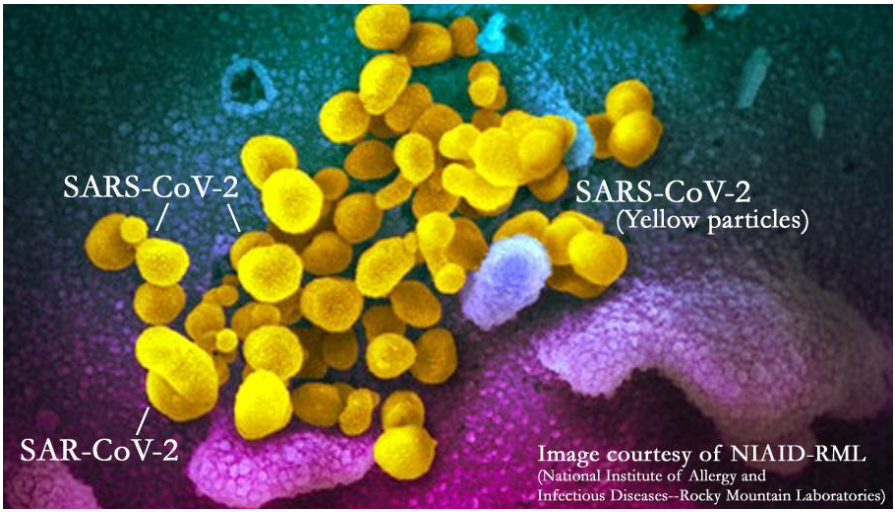
[31] Belshaw, "What Is Special about RNA Viruses?" He notes that the only virus humanity has managed to eliminate—smallpox—is a DNA virus.

[32] Peiris, "Coronaviruses," 588.

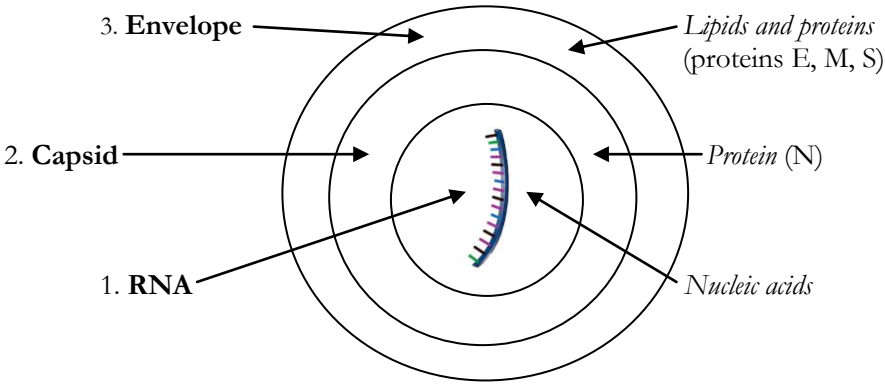
[33] "Coronaviridae," 440.

[34] Culfari, "The Size of SARS-CoV-2 and Its Implications."

Coronaviruses are not only small, but *pleomorphic*—a Greek derived compound meaning 'more' (*pleō-*) in 'form' or 'shape' or 'appearance' (*-morphē*). In viruses this refers to the irregularity among virus particles of the same kind. Although coronaviruses vary in shape they most often are spherical, as depicted in the image and illustration earlier. Some sense of the variability can be gained by looking at images derived from an electron microscope, like this one:



RNA (the viral nucleotides) is the base component of a coronavirus virion, but not the only component of the complete virion structure. The shape of a coronavirus is a reflection of its composition. The structural makeup of coronaviruses can be simply depicted (and the numbering is from the innermost area to the outermost, with structural names to the left and composition to the right):



Possessed of a Protein Shell (*Capsid/Nucleocapsid*)

The *capsid* (Latin, 'cage' or 'carriage') is the first layer around the RNA core. It is made entirely of proteins. The following illustration, though not of a coronavirus, displays the nature of the capsid and its relation to the viral RNA.

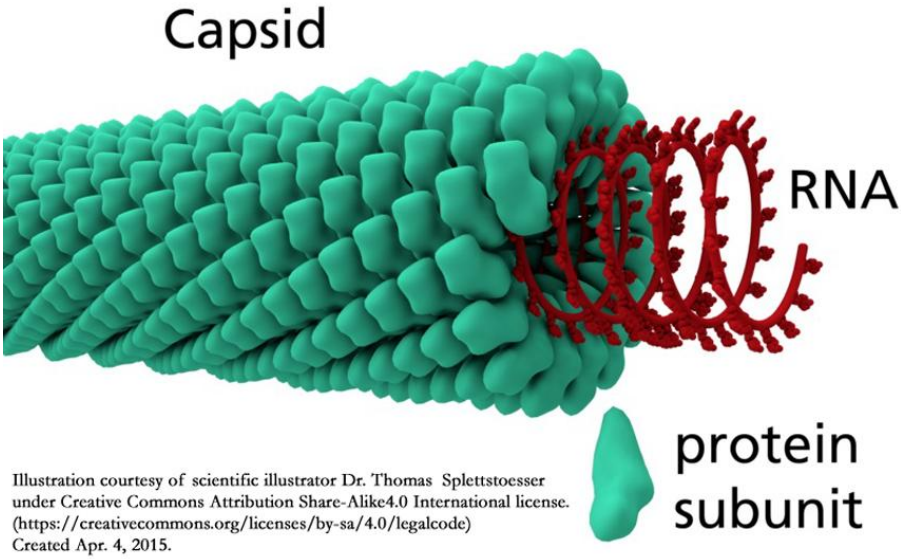
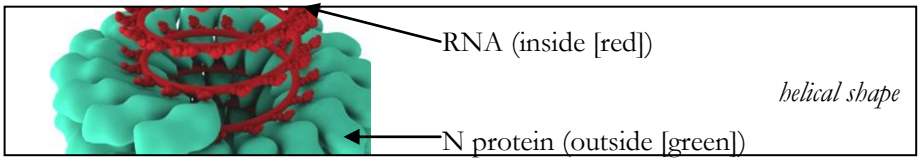


Illustration courtesy of scientific illustrator Dr. Thomas Splettstoesser under Creative Commons Attribution Share-Alike4.0 International license. (https://creativecommons.org/licenses/by-sa/4.0/legalcode) Created Apr. 4, 2015.

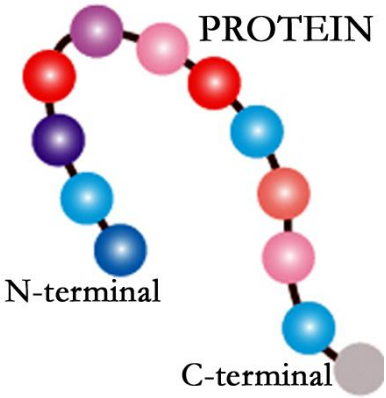
The capsid plus the RNA nucleotides are collectively referred to as the *nucleocapsid*—the 'cage' or 'carriage' (*-capsid*) of the nucleus (*nucleo-*), the viral RNA. It is not unheard of to find this combination reduced to just the term 'capsid,' but we'll prefer nucleocapsid to keep us reminded of what it is about. Its purpose most obviously is to protect the viral genome by completely covering it with protein. However, the nucleocapsid also helps in viral replication and transmission.

The nucleocapsid protein is commonly referred to simply as 'N.' It is the only protein present in the nucleocapsid. The shape of the nucleocapsid is helical (i.e., like a spiral) and 9–11 nm in diameter.[35] Using the previous image, cropping and reorienting it, may help us focus on this helical shape with the N protein binding to the RNA nucleic sequences it surrounds like a cap:



[35] Peiris, "Coronaviruses," 588.

To better understand this protein we must delve a little deeper. A term frequently encountered in genomic literature is *domain*. It is a common enough English word and means 'area,' whether physically, as in land, or metaphorically, as in a mastery of a subject or skill. The basic sense is retained in genomics. A domain is a *region* of genetic code or a *sequence* of amino acids in a protein.



Proteins also have ends, and these are named *terminals*, so-called because the end residue—an individual amino acid—is not part of a peptide bond. The terminal at the start of a protein is called the *N-terminal* and the one at the other end is the *C-terminal*.[36] It is conventional to write the sequence of a protein from N-terminal to C-terminal.

A *terminal domain* is that region or sequence at one end or the other of the protein. The area between these tends to be assigned different names but links the other two. Domains are further distinguished as *structured* or *unstructured* and we shall shortly see what that means. For our purposes we need not know every nuance of domains; this basic grasp will suffice.

The N protein in coronaviruses has two structural domains—that is, a run of amino acids in independently folded sections each with a discrete function.[37] One is named the N-terminal domain (NTD) and the other is called the C-terminal domain (CTD). In addition, there exists between these two domains a third one, intrinsically disordered, variously known as an *intrinsically disordered region* (IDR), the *linker region* (LKR), or *RNA-binding domain* (RBD), even though in various coronaviruses all three domains may bind RNA.[38] The configurations of these domains look different, but we need not at this point examine those differences. Instead, we can simply set out their order (headed by numbers often assigned):

N1b *N-terminal* *domain* (NTD)	*Linker* *region* (LKR) *[aka RNA-binding* *domain* (RBD)*]*	N2b *C-terminal* *domain* (CTD)

[36] The 'N' and 'C' refer to aspects of biochemistry we need not get into here.

[37] Kahl, *The Dictionary of Genomics*, III, 1797, defines a protein domain as "A run of amino acids in a protein (typically from 40 to 700 residues), that folds into a distinct, compact and stable three-dimensional niche independently of other such structures. A protein domain usually has a specific function (e.g., catalysis, binding, substrate modification, to name a few)."

[38] On these domains, see McBride, Van Zyl, and Fielding, "The Coronavirus Nucleocapsid" (especially p. 2992); also Yadav et al., "Role of Structural and Non-Structural Proteins," 4–5.

Of course, the overall structure of the N protein is not as simple as these three domains sitting side-by-side.[39] However, further specification can be reserved for the next chapter as we target the coronavirus causing COVID-19.

At this point it matters that we again turn our attention to some basic ideas about proteins. It is not just N proteins that have domains; this is a general feature of proteins. Two types of domains are distinguished. The first, *structured domains*, as noted above, often fold independently and assume a specific three-dimensional structure to conduct their business. However, there are also often functional segments absent any defined three-dimensional structure and these are termed *intrinsically disordered regions* (IDRs), or *intrinsically disordered domains* (IDDs).[40] Both kinds of domains are found in coronaviruses.

Talking about 'domains' and further specifying them as 'structured' or 'intrinsically disordered' is somewhat jargonistic, but such is not merely the way of science but of virtually all specialized endeavors; members of a community develop their own vocabulary and ways of expression. Here is the key:

- a *domain* is a specific region or protein sequence associated with a predictable function—a place known for what it does;
- a domain is *structured* if it has a particular and predictable structure associated with its function (i.e., it looks a certain way); and
- a domain is *intrinsically disordered* if it doesn't have a particular and predictable structure associated with its function (i.e., its look changes).

Now, let's get back to looking at this situation with the N protein.

We have been referring to the N protein domains as 'functional.' From a practical standpoint we are relatively more interested in what the N protein *does* than how it *looks*—but its function and its form are associated.

Overall, the N protein plays a primary structural role, namely packaging the RNA genome, as we have seen.[41] Binding of the N protein with the viral RNA produces a *ribonucleoprotein particle* (RNP). N also has roles in regulating viral

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[39] For a brief history of the study of the nucleocapsid protein, see Chang, "The SARS Coronavirus Nucelocapsid Protein," 41. The difference in terminology—'region' or 'domain'—need not be confusing. The distinction between a 'region' and 'domain' may seem a subtle one because both refer to physical sections of a genetic sequence. Regions are "contiguous functional elements" (De la Cru et al., "Gene, Region, and Pathway Level Analysis," 3), such as genes, and a basic unit in genomic analysis. Though not formally synonyms the terms are often used synonymously, for example, defining protein domains as 'individual regions of the genome which map to individual protein domains' (see Richardson et al., "A Protein Domain," 2).

[40] To get at this subject more, see Gao et al., "Intrinsic Disorder in Protein Domains"; also Van der Lee et al., "Classification of Intrinsically Disordered Regions and Proteins"; and, Lieutaud, "How Disordered Is My Protein?"

[41] Chen et al., "Structure of the SARS Coronavirus Nucleocapsid," 1076, put it succinctly: "The nucleocapsid protein of SARS-CoV packages the viral RNA to form a helical capsid and is essential for viability."

RNA replication, transcription, translation, and so on. In some coronaviruses it has been linked to a significant increase in production of virus-like particles.[42]

Each of the domains have important functions, which we can summarize:

N-terminal domain (NTD)	*Linker region* (LKR)	*C-terminal domain* (CTD)
↓	↓	↓
RNA binding (to RNA containing TRS or cTRS)[43]	uncertain, but RNA binding involvement[44]	RNA binding (as a dimerization domain)[45]

Note: the NTD and CTD domains presumably fold independently into separable RNA binding domains.

The *nucleocapsid NTD* is often described in its folded shape as looking like a hand with fingers, palm, and wrist. In coronaviruses the NTD of the nucleocapsid shows some common characteristics (e.g., associating with the 3′ end of the viral RNA genome), but also divergence in sequence and length. Functionally, NTDs assist viral RNA in achieving its structure.[46]

The *nucleocapsid CTD* likewise appears to have a role in RNA packaging and functions as a RNA-binding domain. However, it works differently than NTD. CTD acts as a dimerization domain. *Dimerization* is a process that facilitates binding site activity (dimerization being joining two molecules of similar composition into a single polymer called a 'dimer'). The CTD has been reported as capable of greater affinities than the NTD in binding to single-stranded RNA, single-stranded DNA, and double-stranded DNA.[47]

The *nucleocapsid LKR* remains the least understood of the three major parts. Its nature as 'intrinsically disordered' means it is flexible rather than presenting a stable three dimensional structure. Studies have sometimes reached divergent conclusions about some of what the LKR does, but it has been implicated in transcription, translation, post-translation activity, and cell-signaling.[48]

Finally, if it is wondered at that so much attention in a relatively small chapter has been accorded to the N protein, it may be worthwhile to heed what virologists Hubert Laude and Paul Masters observed as far back as the mid-1990s: "The nudeocapsid protein appears to be the most abundant viral polypeptide in coronavirus-infected cells during all stages of infection."[49]

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[42] Hasöksüz, Kiliç, and Saraç, "Coronaviruses and SARS-CoV-2," 550.
[43] Grossoehme et al., "Coronavirus N Protein N-Terminal Domain (NTD)," 545. TRS = transcriptional regulatory sequence; cTRS = complement transcriptional regulatory sequence.
[44] See, for example, McBride, van Zyl, and Fielding, "The Coronavirus Nucleocapsid," 2994.
[45] Chen et al., "Structure of the SARS Coronavirus Nucleocapsid," 1076.
[46] McBride et al., "The Coronavirus Nucleocapsid," 2992–94.
[47] Chen et al., "Structure of the SARS Coronavirus Nucleocapsid," 1077.
[48] McBride et al., "The Coronavirus Nucleocapsid," 2994.
[49] Laude and Masters, "The Coronavirus Nucleocapsid Protein," 148.

Surrounded by an Outer Wrapping (*Lipid Membrane 'Envelope'*)

While not all viruses have a surface covering over the capsid, coronaviruses do. Thus they are termed *enveloped*. Let us look at a new illustration:

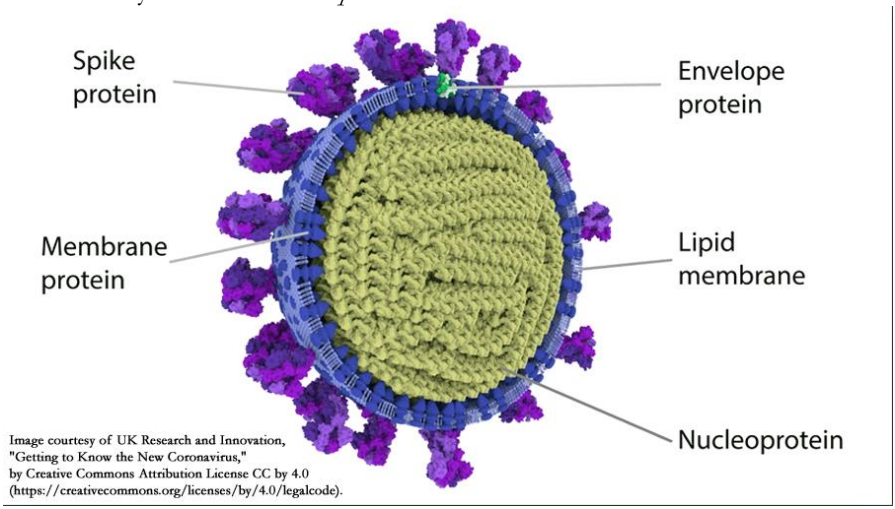


Image courtesy of UK Research and Innovation, "Getting to Know the New Coronavirus," by Creative Commons Attribution License CC by 4.0 (https://creativecommons.org/licenses/by/4.0/legalcode).

While the capsid is made up of proteins, the surface *envelope* is a membrane composed of lipids (from a root meaning 'to adhere')—a basic building block for cells.[50] Put in formal scientific terms, "The association of the nucleocapsid (N) protein with the genomic RNA forms the helical nucleocapsid that is surrounded by an icosahedral structure composed of the viral membrane (M) protein."[51] In plainer language, as we've seen, the N protein plus the viral RNA makes a spiral-shaped nucleocapsid; this now is itself covered by an enveloping structure made up of 20-sided geometric faces ('icosahedral,' from the Greek word for 'twenty'), each face being an equilateral triangle.[52]

Icosahedral Geometry

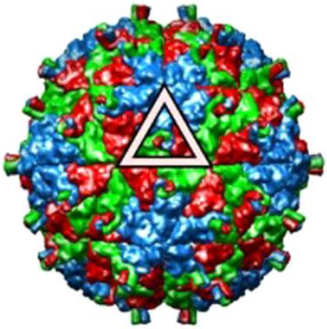


Image courtesy of Twarock and Luque, "Structural Puzzles in Virology Solved with an Overarching Icosahedral Design Principle," *Nature Communications* 10 (2019) 4414. https://doi.org/10.1038/s41467-019-12367-3, by Creative Commons Attribution 4.0 International license (https://creativecommons.org/licenses/by/4.0/legalcode). This image is excerpted from Fig. 4 of the article, where it is "a" (which has been removed); additionally a title has been added.

[50] On the general subject of lipids, see Van Meer, Voelker, and Feigenson, "Membrane Lipids."
[51] "Coronaviridae," 440.
[52] Louten, *Essential Human Virology*, 22.

The coronavirus envelope involves three of the four important structural proteins:[53]

- *M* (Membrane) protein defines the shape of the envelope;
- *E* (Envelope) protein is the smallest and only a small portion is included in the envelope membrane; and,
- *S* (Spike) protein protrudes from the envelope.

The *M (Membrane) protein* is the most abundant—and the most interactive. It interacts with both of the other structural envelope proteins (E and S), and with N. Along with the N protein it is most responsible for the assembly of a coronavirus.[54] M and N interactions stabilize the nucleocapsid. The M protein has three domains:

N-terminal ectodomain	*triple spanning transmembrane domain*	*C-terminal endodomain*

The M protein is a 'triple spanning transmembrane,' meaning it spans the entire virion membrane multiple times. The N-terminal and C-terminal domains may be expressed on either side.[55] The C-terminal endodomain, as the name suggests, is 'internal' (*endo*), in the virion interior where it contacts the nucleocapsid. It constitutes more than half of the M protein.[56]

The *E (Envelope) protein* is not only the smallest of these proteins (76–109 amino acids)[57] but the most variable[58] and the one least understood. E is known to play a pivotal role in formation of the virus envelope even though only a small portion of it is incorporated there. Its interactions with M produce and release coronavirus-like particles (VLPs).[59] The majority of it is localized where intracellular trafficking occurs (the Endoplasmic Reticulum (ER), Golgi-complex (GC), and Endoplasmic reticulum-Golgi intermediate compartment (ERGIC)—and especially the first two). There it participates in virus assembly and 'budding' (described later).[60] The E protein has three basic domains:

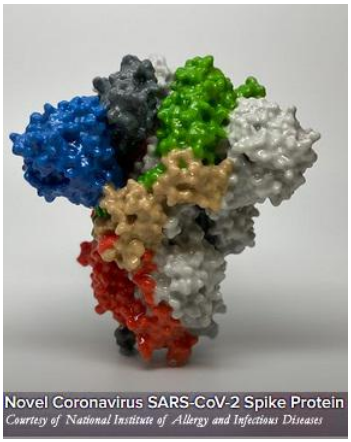
N-terminal domain (NTD)	*transmembrane domain* (TMD)	*C-terminal domain* (CTD)

[53] For a brief introduction, see Schoeman and Fielding, "Coronavirus Envelope Protein," 2.
[54] Hurst, Koetzner, and Masters, "Identification of In Vivo-Interacting Domains," 7221, remark, "The assembly of coronaviruses is driven principally by homotypic and heterotypic interactions between the two most abundant virion proteins, the membrane protein (M) and the nucleocapsid protein (N)."
[55] Locker et al., "Membrane Assembly of the Triple-Spanning Coronavirus M Protein," 21911.
[56] Hurst, Koetzner, and Masters, "Identification of In Vivo-Interacting Domains," 7221; also see Wang, Grunewald, and Perlman, "Coronaviruses," 7.
[57] Schoeman and Fielding, "Corona Virus Envelope Protein," 2.
[58] Wang, Grunewald, and Perlman, "Coronaviruses," 4–5.
[59] Maeda et al., "Membrane Topology of Coronavirus E Protein."
[60] Schoeman and Fielding, "Corona Virus Envelope Protein," 2–3.

Distinguished by Spikes on the Viral Surface

The *S (Spike) protein* is the third major structural protein associated with the coronavirus surface membrane. It is easily the most famous part of the coronavirus to the general public. The S protein is a *Class I fusion protein*, a classification based on its structure, which is as a *trimer* (*tri-* 'three'; *-mer* 'parts') in structure both before and after fusion with a host cell, which happens early in infection but can also occur in late infection.[61] Formally, it can be described as, "a clove-shaped trimer with three S1 heads and a trimeric S2 stalk."[62]

To better understand this let's examine a close-up image.



The colored illustration permits visualizing the S protein as a *quaternary structure*—i.e., one where protein subunits join in a closely packed arrangement. *Monomers* (small single chemical units) are assembled into the protein's distinctive *trimeric structure* (i.e., the three identical monomers join in a compound; see 'trimer' above). The S protein is a *homo-trimer*, meaning it is made of three identical units.

In most, but not all coronaviruses, S is divided through protease cleavage (discussed later) into two subunits, blandly labeled S1 and S2.[63] In the pre-fusion state (i.e., its normal state, before viral infection of the host cell), S1 predominates at the top, forming a sort of cap, while S2 predominates at the base (or 'stalk') of the protein.

Before we dive a little deeper, here is the S protein structure:

N-terminal domain (NTD)	*transmembrane domain* (TMD)	*C-terminal domain* (CTD)

At the N-terminus is a signal peptide, followed by the S1 subunit, and the S2 subunit. The NTD extrudes from the cell (i.e., is 'extracellular'). The TMD is anchored in the membrane itself. The CTD is both short and intracellular.[64]

The *subunit 1* (S1) has both NTD and CTD, either of which can be a receptor-binding domain (RBD).[65] S1 binds to receptors on the cell being infected.[66] In human coronaviruses this binding typically is through the C-terminal of S1.[67]

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[61] Wang, Grunewald, and Perlman, "Coronaviruses," 4. On the general topic, see White et al., "Structures and Mechanisms of Viral Membrane Fusion Proteins."
[62] Li, "Structure, Function, and Evolution of Coronavirus Spike Proteins," 238.
[63] Wang, Grunewald, and Perlman, "Coronaviruses," 4.
[64] Huang et al., "Structural and Functional Properties," 1141–42.
[65] Tang et al., "Coronavirus Membrane Fusion," 3.
[66] Huang et al., "Structural and Functional Properties," 1142–43; also, Juraszek et al., "Stabilizing the Closed SARS-CoV-2 Spike Trimer," 2.

The *subunit 2* (S2) has both TMD and CTD, among other things. It is responsible for membrane fusion.[68]

S interacts with M, which keeps S in a particular area (ERGIC) that constitutes an important sorting station in the pathway by which cells secrete proteins.

In coronaviruses most of the spike protein (S) shows three characteristics:

- S is *exposed on the outside of the virus* (through the S1-NTD).
- S has a *short transmembrane domain* (the CTD).
- S is *followed by a short cytoplasmic tail.*[69]

The exposure of S on the outermost side of the virus gives coronaviruses their distinctive profile. In technical terms, the S protein is said to have 'a large *ectodomain* and a small *endodomain*.'[70] This just means most of it extends outside the virus (*ecto-* 'outer' or 'external'). Thus only a small remaining portion is inside the virus (*endo-* 'within').

Spike protein has a short transmembrane domain (the CTD). This single pass membrane serves to anchor the S protein in the viral membrane.

The S protein is followed by a short cytoplasmic tail. This is comprised of linked amino acids ('residues') in two distinct sections. The section furthest from the membrane projects into the cytoplasm. The cytoplasmic tail plays an important part in interacting with secretory pathways.[71]

Importance of S Protein

The importance of the spike protein can scarcely be over-exaggerated. It is the means by which coronaviruses latch on to host cells, thus initiating infection. This one function makes S the primary determining factor for a coronavirus' range across species because of its broad ability to infect many types of cells (called 'tissue tropism').[72] The crucial importance of the S protein is captured by researcher Fang Li, who remarks, "In addition to mediating virus entry, the spike is a critical determinant of viral host range and tissue tropism and a major inducer of host immune responses."[73]

Virologists Bernard Delmas and Hubert Laude reported in 1990 that, "The spike glycoprotein has been shown to mediate attachment of virions to the host cell receptor, to be involved in cell-to-cell fusion, to induce neutralizing antibodies, and to bear virulence determinants."[74]

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[67] Wang, Grunewald, and Perlman, "Coronaviruses," 4.

[68] Huang et al., "Structural and Functional Properties," 1142–44.

[69] Woo et al., "Coronavirus Genomics and Bionformatics Analysis," 1809; Huang et al., "Role of Structural and Non-Structural Proteins," 1141. Also see Yadav et al., "Role of Structural and Non-Structural Proteins," 4.

[70] Wang, Grunewald, and Perlman, "Coronaviruses," 4.

[71] Cattin-Ortolá et al., "Sequences in the Cytoplasmic Tail." Also see Sadasivan, Singh, and Das Sarma, "Cytoplasmic Tail of Coronavirus Spike Protein Has Intracellular Targeting Signals."

[72] Wang, Grunewald, and Perlman, "Coronaviruses," 6.

[73] Li, "Structure, Function, and Evolution of Coronavirus Spike Proteins," 238.

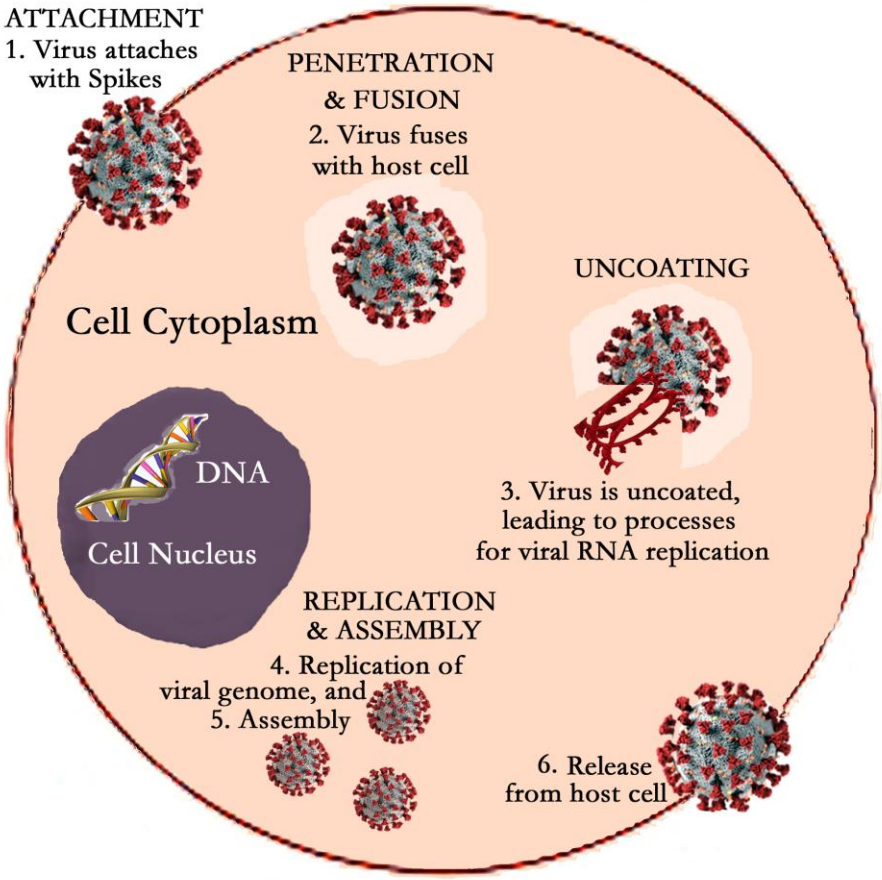
[74] Delmas and Laude, "Assembly of Coronavirus Spike Protein," 5367.

The Viral Cycle

The coronaviruses can be described simply as self-replicating codes dependent on host mechanisms. *Replication* is their *raison d'être*—their reason for being. With respect to replication six distinct stages can be identified.

1. *Attachment*—the virus must come into contact with a host cell.
2. *Penetration and Fusion*—the virus must enter the host cell and fuse.
3. *Fusion and Uncoating*—once inside, the virus coating over the genomic RNA must be removed.
4. *Replication*—the RNA issues instructions to reproduce itself.
5. *Assembly*—newly produced viral material is packaged.
6. *Release*—the newly packaged virus replicates are set loose outside the host cell to become virions ready to infect other host cells.

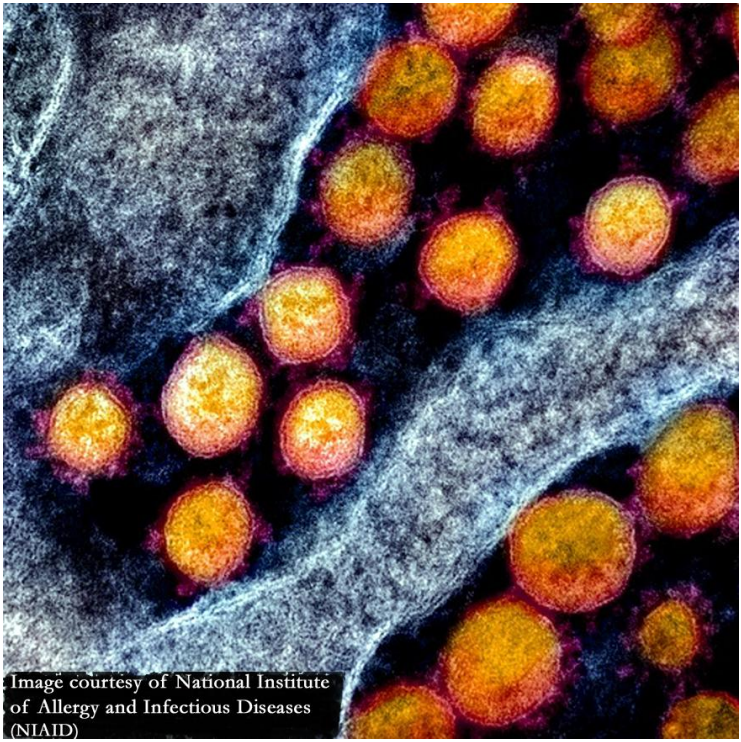
Each of these needs elaboration, but let's begin with an illustrative overview.



(The above overview depicts the most famous avenue of attachment and penetration. Note that there is overlap between the steps; the cycle is a fluidly continuous one.)

1–2. Attachment and Penetration

Naturally, our interest lies mostly in the coronaviruses as infectious agents. A picture, they say, is worth a thousand words, so let's start with one.



The invading viral cells in this image are those of the coronavirus that causes COVID-19.

The life cycle success of a coronavirus, which depends on using a host cell's resources and mechanisms for viral survival, begins with an all-important need to attach and enter a life-giving host. Coronaviruses, as a family, attach to and enter host cells in one or another of three ways:

- via host receptors that permit fusion with the host cell membrane (*receptor-mediated plasma membrane fusion*);
- via host receptors that absorb the virus through an inward budding pouch of cell membrane that then dissolves within the host cell (*receptor-mediated endocytosis*); or,
- via the host's own antibodies (*antibody-dependent viral entry*).

For both fusion and endocytosis, receptor proteins on the surface of host cells are crucial for virus attachment.[75]

[75] Zhang et al., "Molecular Mechanism of Interaction between SARS-CoV-2 and Host Cells," 2.

The first way named above is formally termed *receptor-mediated plasma membrane fusion*. Let's examine the terminology: 'receptor-mediated' simply means that the host cell's own process of receiving things from outside the cell is used against it. The 'plasma membrane,' also called the cell membrane, is the boundary surface of the cell that separates what is inside it from what is outside it. Obviously, the virus outside the cell wants to get inside. 'Fusion' is the process of joining the virus to the host cell. So when these elements are put together we have a virus using the way cells relate to the outside environment to join with it along its outer boundary.

There are three fundamental parts to this process:

- *initial attachment* to a host cell's receptor sites;
- *entry into the cell trafficking system* (*endosomes*); and,
- *fusion* between the virus and the host cell.

A coronavirus, just like a host cell, needs ways of communicating with the environment outside. These doors or windows to the outside—receptors—are as vitally important to the virus as to the host cell. As microbiologists Joe Grove and Mark Marsh put it, the initial interactions between virus and host "serve primarily to give a virus an initial catch-hold from which it can then recruit specific receptors that drive the reactions leading to entry."[76]

Coronaviruses need some way to attach to a host cell. In simple terms, they need to find a door or window in order to break in. The host cell receptors serve as such. The S (Spike) protein acts as the viral strongman to force entry into the unwilling host cell.

Earlier in the chapter we saw that the S1 subunit of the S protein—the outermost portion of the protein—includes a receptor-binding domain (RBD). However, the exact site of the RBD within that unit varies among coronaviruses. In most human coronaviruses (e.g., HCoV-229E, HCoV-HKU1, HCoV-NL63, SARS-CoV, and MERS) it is present in the C-terminal of S1.[77]

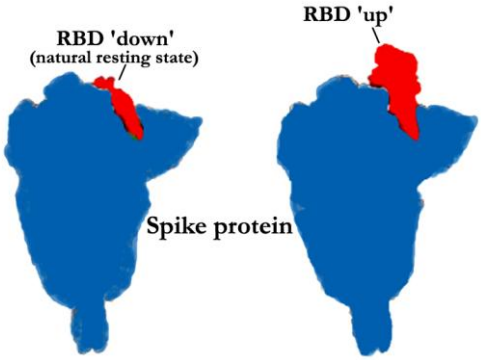
Exactly as the name says, the RBD binds the coronavirus to a *receptor*—a place on the surface of a cell where it can 'receive' information from other cells (see illustration, second following page). The RBD has short, specific areas designed to facilitate *glycosylation*—a chemical reaction between the RBD and a receptor molecule of the host cell. The RBD glycosylation site secretes bits of RBD through glycosylation that then fold and bind to the host cell receptor.

The S protein chemically recognizes a very specific kind of receptor, which can vary among the coronavirus family.[78] We are here interested in those human coronaviruses that bind to a receptor labeled *ACE2* (angiotensin-converting

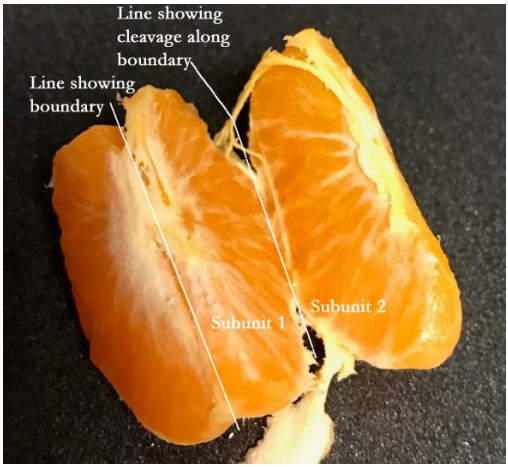
[76] Grove and Marsh, "The Cell Biology of Receptor-Mediated Virus Entry," 1071.
[77] Wang, Grunewald, and Perlman, "Coronaviruses," 4.
[78] V'koski et al., "Coronavirus Biology and Replication," 155.

enzyme 2). The ACE2 receptor is preceded by 'h' to indicate specifically 'human' ACE2 receptors, and so our focus will be on hACE2 receptors.

The RBD of the virus S1 subunit rises to a standing, or 'up' position, exposing the receptor-binding site, which then can bind to a pocket on the hACE2 receptor. Before fusion with a host, the S protein is in a resting state. The RBD rests at the top of the spike's trimeric structure. Three copies of the RBD are surrounded by three copies of the NTD. In the resting, prefusion state the RBDs lie flat—metaphorically 'closed for business.' One or more of these RBDs must open by lifting up in order to expose the receptor-binding site required for fusion.[79]



"Viral fusion," as researchers Yuan Huang and colleagues explain, "refers to fusion of the viral membrane and host cell membrane, resulting in the release of the viral genome into the host cell."[80] As noted earlier, coronavirus S proteins belong to the class I fusion proteins—a grouping based on *how* they fuse. In this class fusion is primed for fusion-activation by *proteolytic cleavage* (the term means using an enzyme on a protein ('proteolytic') to break a bond in it ('cleavage')). Across the different groups of coronaviruses (discussed later) this activation varies.[81] Thus, when and where fusion occurs varies across coronaviruses.[82]



The concept of cleavage can be easily grasped from ordinary experience. The illustration at left shows a common orange. At its left side, in a pre-cleavage state, we can see a boundary exists between slices, which we can call 'subunits' of the orange. In the center of the image we see another line, this one showing where cleavage is separating the two slices (subunits 1 and 2). The agent that breaks the subunits apart is unchanged by the act—

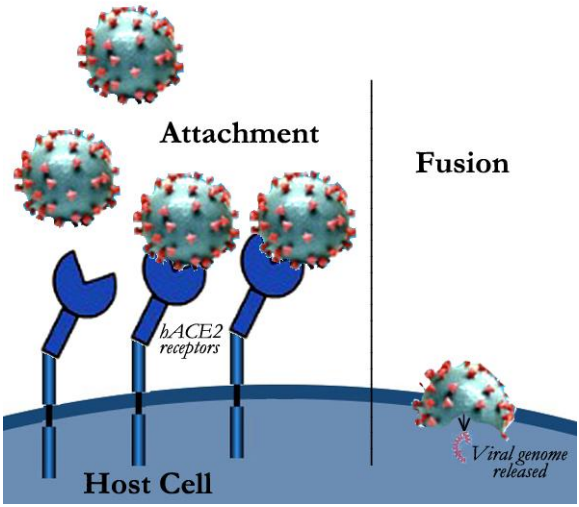
[79] Ke et al., "Structures and Distributions of SARS-CoV-2 Spike Proteins," 498.
[80] Huang et al., "Structural and Functional Properties," 1144.
[81] Belouzard, Chu, and Whittaker, "Activation of the SARS Coronavirus Spike Protein," 5871.
[82] Jackson, et al., "Mechanisms of SARS-CoV-2 Entry into Cells," 1.

just as in nature when an enzyme works to sever bonds.

The cleavage occurs, as one would expect, along the boundary between S1 and S2 at what is termed the S1/S2 site.[83] However, in some coronaviruses (e.g., SARS-CoV) cleavage also can occur not far from the S1/S2 site in the CTD of the S2 subunit.[84] Typically cleavage happens in the area of the viral fusion peptide. This becomes exposed with the changes in the S protein wrought by cleavage. Then, after insertion into the host cell, it initiates fusion itself.[85]

Proteolytic cleavage involves an enzyme capable of breaking down such bonds, called a *protease*. This might be either a trypsin-like protease or furin. Whichever it might be, the protease breaks the bond between subunits preparatory to fusion. *Trypsin* or *trypsin-like protease* breaks the peptide bond between a carboxyl group in a protein and an adjacent amino group.[86] *Furin* seems even more prominent in the kinds of coronaviruses causing the most harm to people, though furin cleavage sites are common throughout the coronavirus family. What makes furin particularly important in infection by the more deadly coronaviruses is that it has a wide distribution range in the human body—a reality that may contribute to how easily and rapidly such infections spread.[87]

We have seen the S1 subunit's important role in attachment. But S2 has its role as well. The S2 subunit, recall, is responsible for membrane fusion. It contains the fusion peptide responsible for fusing the coronavirus virion and host cell.[88] Thus, in our little drama, while S1 plays the starring role in the first act (attachment), in the second act (fusion) S2 emerges into the spotlight. Upon cleavage of S2, a *fusion peptide* (FP) of the S protein—only a 15–25 amino acids sequence—is inserted into the host cell membrane. This triggers the actual event of fusion.[89]



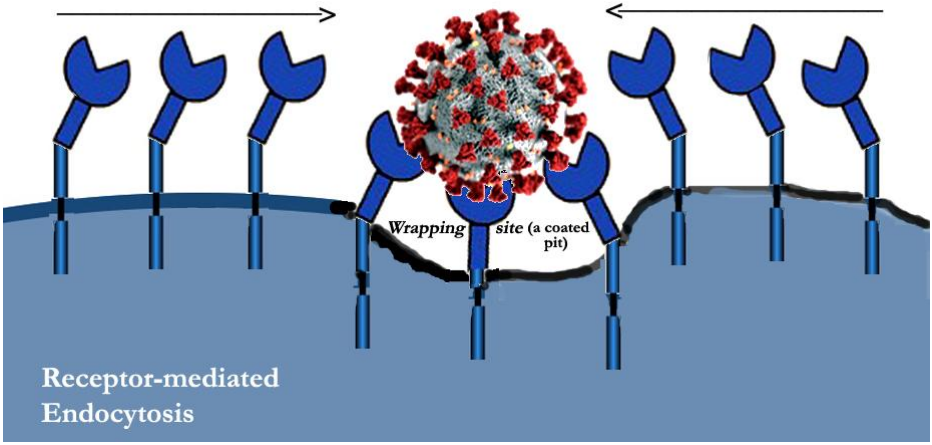
[83] Boopathi, Poma, and Kolandaivel, "Novel 2019 Coronavirus Structure," 2.
[84] Wu and Zhao, "Furin Cleavage Sites," 1.
[85] Boopathi, Poma, and Kolandaivel, "Novel 2019 Coronavirus Structure," 2.
[86] See, for example, Bertram et al., "Cleavage and Activation."
[87] Wu and Zhao, "Furin Cleavage Sites," 2–3. Also see Whittaker, "SARS-CoV-2 Spike and Its Adaptable Furin Cleavage Site." Also see Wang, Grunewald, and Perlman, "Coronaviruses," 7.
[88] Wu and Zhao, "Furin Cleavage Sites," 1; also see Li, "Structure, Function, and Evolution of Coronavirus Spike Proteins," 238.
[89] Tang et al., "Coronavirus Membrane Fusion," 4; Samavati and Uhal, "ACE2."

A second way viruses can infect is through *receptor-mediated endocytosis*.[90] As in the first way, host cell receptors—the entry way for receiving things from outside the cell—are used by the virus for illicit purposes. The word 'endocytosis' is a compound form of Greek terms that we can re-sort in English. It means 'a condition' (*-osis*) of being 'within' (*endo-*) a 'vessel' (*-cyt-*). In short, receptors of the host cell are used to sneak in the virus within a vessel.

Let's start with a basic sense of what is happening. *Endocytosis* is a very fundamental and essential process for a cell. It is a cell's digestive process—it provides a way to take in large molecules from outside the cell and then break them down inside the cell. Yet endocytosis does even more, having a role in many aspects of cell life, including in the defense against invasive organisms.[91] Viruses using receptor-mediated endocytosis are hijacking a key cell process.

In endocytosis the virus makes contact with the host cell's receptors. These collect along a *wrapping site* on the host cell membrane. Receptor density at this site increases, the cell membrane sags, and the receptors wrap around the viral particle.[92] This sagging, or folding inward (*invagination*), creates an encircling pocket (*vesicle*). This pocket moves into the host cell cytoplasm along the *endosomal network*, which transports membrane-bound vesicles (*endosomes*).

Host cell receptor density increases along wrapping site.



Please note that cell entry need not be *either* receptor-mediated fusion *or* receptor-mediated endocytosis. Some coronaviruses use both means. As researchers Evgeny Knyaev and colleagues put it, "Viruses can penetrate and infect cells of barrier tissues directly through the cytoplasmic membrane and indirectly by

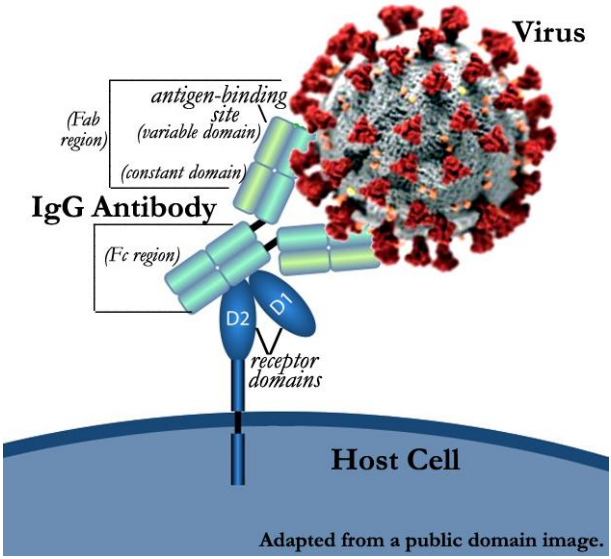
[90] Endocytosis has been noted for all the human coronaviruses. See Bayati et al., "SARS-CoV-2 Infects Cells," 2.
[91] For a history of the study of endocytosis, see Schmid, Sorkin, and Zerial, "Endocytosis."
[92] Gao, Shi, and Freund, "Mechanics of Receptor-Mediated Endocytosis."

using cell endocytosis pathways."[93] In such cases the range of viral infection may be expanded.

3. Antibody-Dependent Viral Entry

The third way of viral entry into a host cell is *antibody-dependent viral entry*. This means that the body's own immune defense is used against itself. *Antibody-dependent enhancement* (ADE) occurs when antibodies fail to neutralize the virus and instead enhance infection. Thus far, at least five different mechanisms have been found to underline ADE, with different viruses employing different mechanisms.[94] ADE is associated with increased severity of illness. Not surprisingly, ADE is found in the more severe coronaviruses.[95]

Antibodies have a region—the *fragment crystallizable* (Fc) *region*—responsible for ensuring a suitable response occurs to the agent (the *antigen*) provoking the immune system. This region of the antibody binds to the virus, while another antibody region binds to specific host cell receptors—*Fc receptors* (FcRs). In this manner the antibody and virus attach to the host cell and kick into action immune system defenses (e.g., phagocytic and cytotoxic cells, which are discussed later).



But when this process goes awry, instead of neutralizing the virus the attached antibody provides open access to the host cell. This FcR mechanism is the one used by coronaviruses such as SARS-CoV and MERS.[96] In this manner ADE increases the rate of infected cells, which results in higher virus production—all termed *extrinsic ADE*. This situation on its own is bad enough, but evidence suggests *intrinsic ADE*—the internalized immune complexes enhancing viral replication—may also occur. The result of this enhanced viral activity is an exaggerated response by the host cell in which damaging cell inflammation can lead to severe damage.[97]

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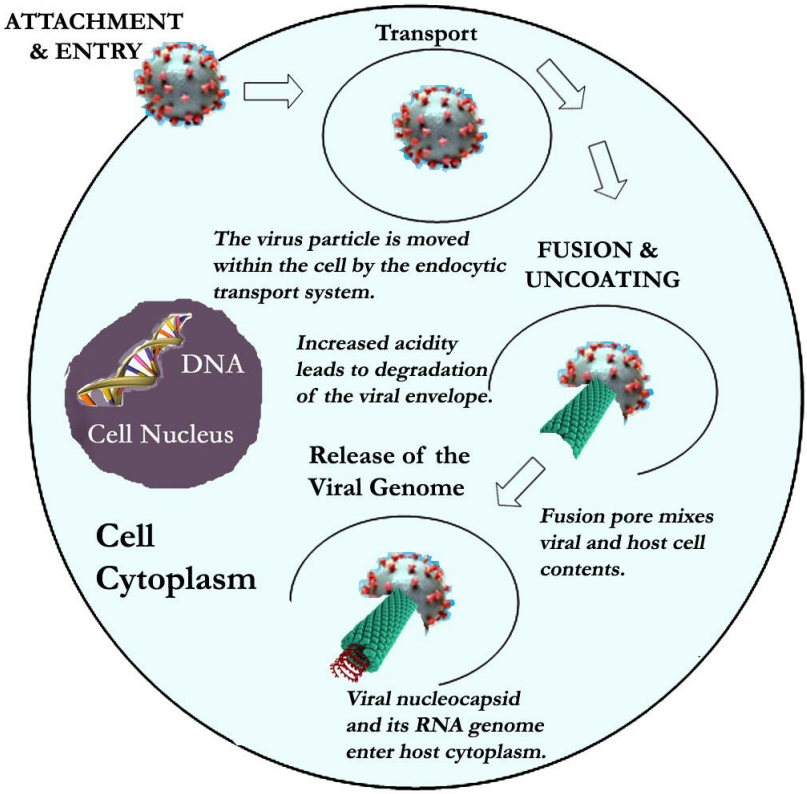
[93] Knyazev, Nersisyan, and Tonevitsky, "Endocytosis," 3.
[94] Wen et al. "Antibody-Dependent Enhancement of Coronavirus," 483.
[95] Lee et al., "Antibody-Dependent Enhancement," 1185.
[96] Wen et al. "Antibody-Dependent Enhancement of Coronavirus," 486.
[97] Kulkarni, "Antibody-Dependent Enhancement," 11, 14.

3. Fusion and Uncoating

The spike protein is the viral fusion protein for coronaviruses. Cleavage produces changes in both subunits that bring the membranes of the virus and host cell together, creating a *fusion pore* that allows the viral genome to reach the cell cytoplasm as the pore expands.[98] More specifically, as the enveloped virus enters the host cell it is transported along pathways inside the host. In this fashion it encounters specialized structures in the host cell called *lysosomes* (from Greek meaning 'to loosen' or 'dissolve'), which degrade the viral envelope.[99]



This degradation process is vital for the virus—exactly what it wants. For some viruses this requires transport to the cell nucleus, but most viruses, including coronaviruses, need only to be transported within the cytoplasm to its fluid matrix known as the *cytosol*, where the viral RNA can do its work.[100]

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[98] Jackson et al., "Mechanisms of SARS-CoV-2 Entry into Cells," 1.
[99] Zhao, Qin, and Huang, "Lysosomal Ion Channels Involved in Cellular Entry," 1–2.
[100] Greber, Singh, and Helenius, "Mechanisms of Virus Uncoating," 54.

Let's look a little closer at what happens. As we have seen, the coronavirus RNA genome is protected by a nucleocapsid. What keeps RNA safe also keeps it confined. This protective covering must be removed for the RNA to issue instructions to the host cell. It has to occur in the cytoplasm because the viral material is too large to pass into the cell nucleus. In essence *uncoating is the reversal of virus assembly*. The virus particle must be disassembled. In a way, this begins already with attachment, where the cleavage of the S protein renders it less stable and hence more susceptible to fusion and uncoating.

We noted earlier that while the spike protein's S1 subunit stars in attachment to the host, S2 stars in fusion. This subunit uses a variety of *motifs*—small regions of the S protein with a recognizable three dimensional structure, each with its own part to play. A leading role is assumed by the *fusion peptide* (FP), a very short (15–25) amino acid sequence, conserved across the coronavirus family, which is inserted into the host cell membrane to trigger the event of fusion. The FP's work is assisted by *heptad repeat regions* (HR1 and HR2), which further drive fusion. (Next to HR2 is the *transmembrane domain* (TM) which anchors the S protein in the viral membrane.)[101]

We saw that attachment and penetration might involve receptor-mediated fusion or receptor-mediated endocytosis. Either way, once the viral particle is in the cell it is moved along a transport system to where degradation—'uncoating' can occur. Uncoating requires the viral envelope be degraded and this involves *lysosomal factors* to trigger uncoating and help induce fusion.[102]

In this intracellular transport system ('endocytic membrane transport pathways'), endosomes carry the viral material. As they move along the pathway toward the lysosomes that will break them down, the pH balance of the system becomes more acidic, contributing to a weakening of the barrier between virus and host cell. This change destabilizes the fusion protein, allowing the fusion peptide (FP) to be inserted. One distinctive feature of coronaviruses seems to be that their FPs are partially exposed at the surface. Once FP is inserted then HR1 and HR2 regions assemble into domains to form a *fusion core*, which pulls virus and host cell for fusion. Fusion itself has two stages. In *hemifusion* the outermost portions of the membranes of virus and host fuse. This is a transient state. In *pore formation* the inner portions as well as the outer are fused so that viral nucleocapsid can be transferred to the host cell cytoplasm through the fusion pore that has been created.[103]

Other factors also seem to be at work. Viral *transmembrane receptors* may serve as 'hotspots' for viral entry, while *cholesterol* in both virus and host cell membranes may pack together, reducing the energy needed for fusion and thus making it more likely. *Calcium* also may play a role through how it stabilizes a structure of FP that facilitates membrane merging.[104]

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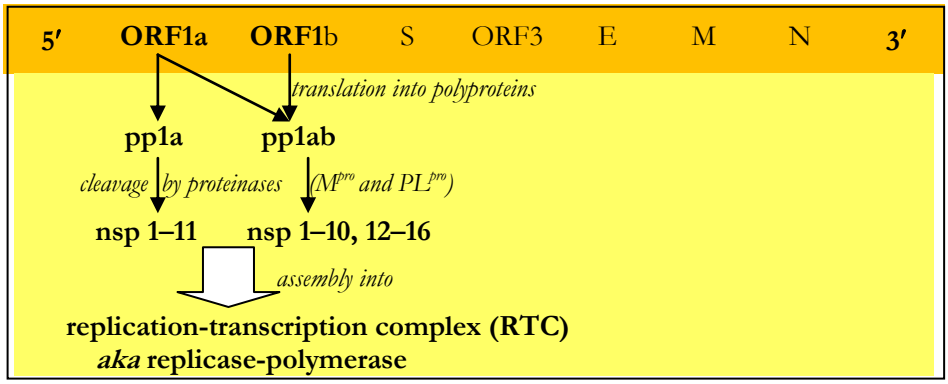
[101] Tang et al., "Coronavirus Membrane Fusion," 4–5.
[102] Zhao, Qin, and Huang, "Lysosomal Ion Channels Involved in Cellular Entry," 2.
[103] Tang et al., "Coronavirus Membrane Fusion," 8–9.
[104] Tang et al., "Coronavirus Membrane Fusion," 10.

4. Replication
(Primary Translation, Transcription, Secondary Translation)

Once the viral genome has been uncoated, processes like transcription and translation—both described in chapter 1—become important. As we noted earlier, viruses literally exist for the sole purpose of reproducing themselves. This is reflected in their genome, where roughly two-thirds of it is devoted to encoding the viral *replicase*—literally, the 'replicating' (*replic-*) enzyme (*-ase*)—that brings about RNA synthesis.[105] A *replicase polymerase* (or *replicase-transcriptase*)—to more formally name this replicase—is an enzyme that catalyzes RNA replication.

But we must start back at the genetic level. So let us reproduce our earlier illustration of the conserved order of seven genes for coronaviruses, but this time we'll highlight the translation process that begins a process that later entails both transcription and translation by adding some new information.



Primary Translation

Now let's explain what we've just seen. In brief, in replicating itself a coronavirus first uses translation of overlapping open reference frames (ORFs), beginning specifically by translating ORF 1a and ORF 1b into polyproteins named pp1a and pp1ab. These are then cleaved into nonstructural proteins (nsp 1–16), which in turn assemble into a replication-transcription complex (RTC).[106]

This brief description needs some complementary additions. A number of enzymes ('replicase polyproteins') have been implicated in coronavirus synthesis. At least four of them result in activities that seem conserved across the coronavirus family. In fact, the coronaviruses appear to be distinctive in the number of enzymes involved in RNA synthesis and modification.[107]

The coronavirus *proteinases* (or *proteases*)—enzymes that catalyze *proteolysis* (the breakdown of proteins)—are used in the cleavage of pp1a and pp1ab, but

[105] Ziebuhr, "The Coronavirus Replicase," 59; Lim et al., "Human Coronaviruses," 2.
[106] Lim et al., "Human Coronaviruses," 4; Ziebuhr, "The Coronavirus Replicase," 59–61 (see notes to Fig. 1).
[107] Ziebuhr, "The Coronavirus Replicase," 62–63.

they are not all of the same kind. A *main proteinase* (M^{pro}) cleaves them at eleven conserved sites; any *accessory proteinase* (PL^{pro} for 'papain-like cysteine proteinase') cleaves them at two or three sites. Across the coronavirus family, all of them appear to have at least one main protease and at least one accessory protease.[108]

As shown in the preceding illustration, the cleavage of pp1a and pp1ab results in sixteen nonstructural proteins (nsp). Like breaking down a chunk of wood to make a set of building blocks, the parts must now be assembled into something meaningfully functional. What is put together is the *replication-transcription complex* (RTC).

Replication-Transcription Complex

Nonstructural protein 1 (nsp1) is rapidly released and targets the host cell's translation machinery. The other fifteen (nsp2–16) compose the RTC itself. Some of these are thought to provide supporting functions (nsp2–11), such as host immune evasion and providing replication cofactors. It is the other five—nsp12–16—that contain the core enzymatic functions involved in *RNA synthesis* (nsp 12 is the RNA-dependent RNA polymerase (RdRp),[109] aided by nsp7 and nsp8), *RNA proofreading* (nsp14), and *RNA modification*.[110] Just mentioned, RdRp catalyzes RNA replication from the RNA template. As the name suggests, this RNA polymerase is dependent on RNA, not DNA (as seen in chapter 1).

Once assembly of the replication-transcription complex (RTC)—or the *replicase polymerase* as it is otherwise called—is complete, the full-length positive strand of viral genomic RNA is transcribed. The transcription forms a full-length negative strand template. This situation now permits the synthesis of new genomic RNAs. It also makes possible the production, from negative *sense subgenomic RNAs* (sgRNAs), templates for generating positive-sense sgRNA, which in turn serves as mRNA[111] for translating various ORFs. In this way the virus can produce structural and accessory proteins.[112] But to make sense of this matter of 'subgenomic RNAs' requires we pause to consider an important feature of coronavirus transcription strategy.

A Nested Set Transcription Strategy

Coronaviruses are coding machines of perpetual motion, always ready to produce copies of their genome and replicate themselves at the expense of a host. Coronavirus researcher Philp V'koski and colleagues put it this way: "During the intracellular life cycle, coronaviruses express and replicate their genomic RNA to produce full-length copies that are incorporated into newly produced viral particles."[113] To maintain this state of readiness and fuel the machinery

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[108] Ziebuhr, "The Coronavirus Replicase," 64–65.
[109] For an introduction to the subject, see Choi, "Viral Polymerases."
[110] V'koski et al., "Coronavirus Biology and Replication," 161–62.
[111] 'Positive sense' means able to function as mRNA. See Payne, *Viruses*, 98.
[112] Lim et al., "Human Coronaviruses," 4; V'koski et al., "Coronavirus Biology and Replication," 162; De Breyne at al., "Translational Control of Coronaviruses," 12503–04.
[113] V'koski et al., "Coronavirus Biology and Replication," 155.

engaged in infection they utilize what has been termed 'a nested set transcription strategy.'

We saw in chapter 1 that transcription is a copying of genetic code that entails a bit of rewriting. In formal terms, "the expression of genes encoding structural viral proteins is mediated via a nested set of 30 coterminal subgenomic mRNAs."[114] A *nested set* is one in which a collection of RNA fragments—all derived from a larger polynucleotide—are characterized both by being of different sizes ('nested'), yet all sharing a common end (hence a 'set'). So what this all means is that the process of a coronavirus making its structural proteins entails a strategy of nested sets by which it uses smaller strands of mRNA (the 3′ coterminal subgenomic mRNAs) to generate many different proteins from the same strand.[115]

Coronaviruses, as is typical of positive-strand RNA viruses, depend on a process of continuous synthesis for genome replication. Although the transcription process in coronaviruses is relatively more complicated than that found in other kinds of RNA viruses (and is itself discontinuous by nature[116]), they are efficient machines at copying and reproducing themselves.[117] When they infect host cells they produce six-to-eight species of virus-specific mRNAs, expressed in different quantities, all making for a 3′- coterminal nested-set structure.[118]

Now we can return to what was said just before this aside and simplify the matter into less-precise but adequate terms: transcription copies the viral RNA in ways to maximize its utility with the host cell machinery. To accomplish this it has to both copy and translate itself into something the host cell machinery can understand. In effect, the viral genome has to express itself as messenger RNA (mRNA) that can instruct the host cell what to do.

Secondary Translation

Remember, where transcription is copying taking place in the same language, translation is moving from one language (the nucleic acids of RNA) to another (the amino acids of proteins). In short, the essence of translation is the viral RNA 'talking' as mRNA to 'message' the host cell how to make the proteins the virus wants. These proteins, of course, are needed for virus replication and they depend on the host cell doing what the virus cannot do on its own.

Researcher Sylvain de Breyne and colleagues put the whole matter very succinctly: "Translation is a key step in gene expression in which the messenger RNA (mRNA) is decoded by ribosomes to produce proteins."[119] These rib-

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[114] "Coronaviridae," 437. An, Maeda, and Makino, "Coronavirus Transcription Early in Infection," 8517, put it this way: "Coronavirus, an enveloped virus containing a large positive-sense single-strand RNA, expresses its genes by producing subgenomic mRNAs."

[115] For the role this plays see V'koski et al., "Coronavirus Biology and Replication"; also see Sawicki and Sawicki, "Coronavirus Transcription."

[116] V'koski et al., "Coronavirus Biology and Replication," 162.

[117] See Sola et al., "Continuous and Discontinuous RNA Synthesis in Coronaviruses."

[118] An, Maeda, and Makino, "Coronavirus Transcription Early in Infection," 8517.

[119] De Breyne et al., "Translational Control of Coronaviruses," 12504.

somes are formally coded as 'S80 ribosomes' and the translation complex depending on them is termed a 'S80 complex.' But though the details may be murky at times as to how all this happens, the basic idea is simple enough. Once the virus has copied itself into the form it needs to communicate effectively, it does just that.

In general, translation entails four steps:

1. *initiation*—recognition of mRNA by the host cell, followed by . . .
2. *elongation*—the ribosome (80S) of the host cell catalyzes the formation of a peptide bond and repeats this process until . . .
3. *termination*—a stop codon is encountered, releasing the peptide from the ribosome; and then . . .
4. *recycling*—the separation into fragments (dissociation) of the ribosome, causing the translation complex (80S complex) to come apart, and the mRNA to be released, readying the stage for the whole process to repeat.[120]

It is in step one that coronaviruses appear to operate somewhat differently than other viruses.

In brief, the transcribed viral RNA undergoes translation to produce viral proteins required for subsequent RNA replication and transcription. In initiation the host cell 'reads' the viral RNA, acting as mRNA, and an 80S translation complex is formed. The ribosome produces proteins until told to stop (elongation and termination). Then the complex breaks apart, the mRNA is released, and the process can start all over again.

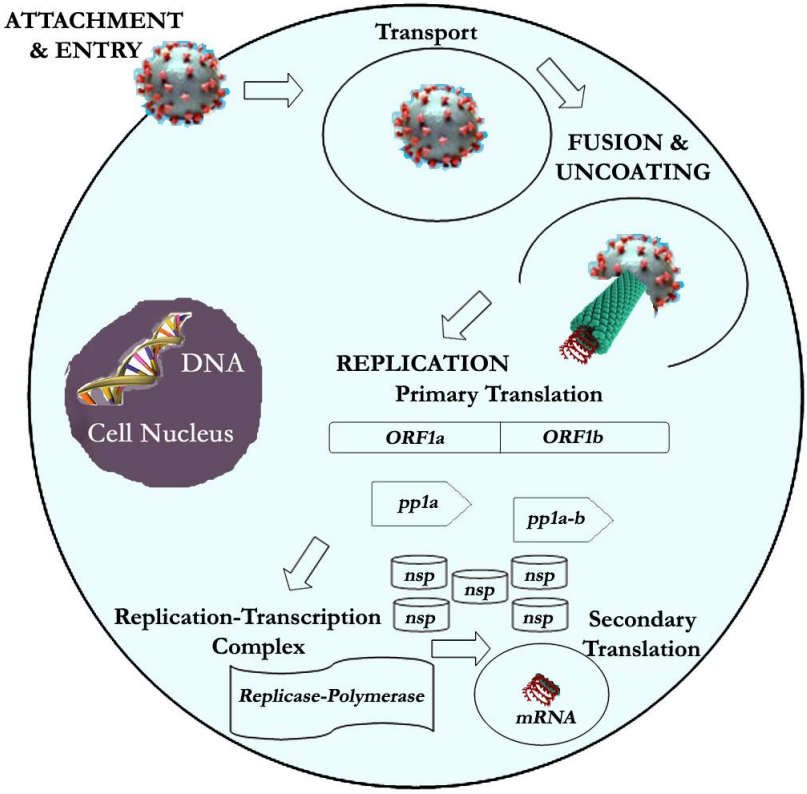
Throughout all of this the viral RNA has to accomplish twin tasks. On the one hand, it must instruct the host cell in making the proteins it wants. On the other hand, it must overcome the resistance of the host cell—its natural inclination to express its own genetic code instead of the virus' genome. Thus, in addition to the things we have been describing—all related to the first of these tasks—the virus is also attending to the second task.

Remember, the host is not completely passive. The host body's immune system is coming to the rescue of the infected host cells. But just as the host recognizes the viral material as foreign and to be rejected, the virus recognizes the host immune response as unfriendly to itself and defends against it. This effort by the virus can be termed *translational control.* A large role in this effort is played by nonstructural protein 1 (nsp1), which as we saw above is cleaved early in the replication process. This protein is expressed in two of the coronavirus family groups (the alphacoronviruses and the betacoronaviruses). In the betacoronaviruses nsp1 is larger, but variable among its members. But despite these variations in size, nsp1 is vital in each instance in inhibiting the host cell's mechanisms for producing its natural proteins. In an instance like SARS-CoV, this entails both direct inhibition of the host cell's initiation of translation, and ac-

[120] Nakagawa, Lokugamage, and Makino, "Viral and Cellular mRNA Translation," 166–67.

tually inducing host cellular degradation of its mRNA. Other viral proteins, such as the nucleocapsid (N) protein also appear to play roles in suppressing the host cell's natural inclinations. [121] While much remains to be learned about how all this works itself out, it is clear that the virus is relatively successful at it.

The illustration below depicts the stages leading up to replication (namely, [1] attachment and [2] entry/penetration, then [3] fusion and uncoating), then the replication cycle itself (primary translation, transcription, and secondary translation).

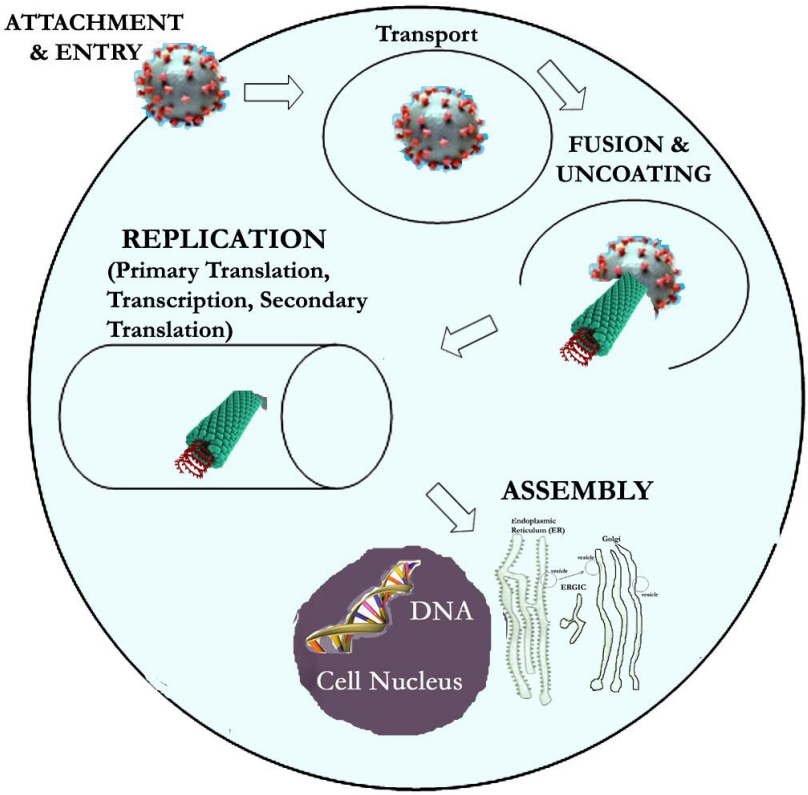


Through the first two-thirds of the stages of the viral cycle, the virus has gone from being an unattached virion to a degraded particle that sets loose its RNA genome as mRNA to repurpose the host cell's functioning. It would not be unfair to see this in some respects as mutually assured destruction—both the virus and the host cell are profoundly changed and the processes employed have been destructive ones. But now the virus has begun to rebuild itself.

[121] De Breyne et al., "Translational Control of Coronaviruses," 12506–15.

5. Assembly

Virologist Keisuke Nakagawa and colleagues point out that, "Besides its role as an mRNA for replicase polyprotein expression, the genome RNA is packaged into progeny virus particles."[122] This observation brings us to the stage of viral assembly in the host cell. Let's start with some visual context.



The parts constructed by the host cell machinery at the direction of the viral mRNA now have to be assembled. This assembly will produce new virus-like particles (VLPs). The nucleocapsid with its genomic material interacts with viral proteins associated with the viral envelope (S, M, and E). In fact, the membrane (M) protein coordinates assembly by gathering the viral envelope components and mediating the interactions with the nucleocapsid. This interaction erects scaffolding for the formation of the viral envelope, complete with the familiar spike (S) protein 'crown.'[123] The viral genome completes the package.

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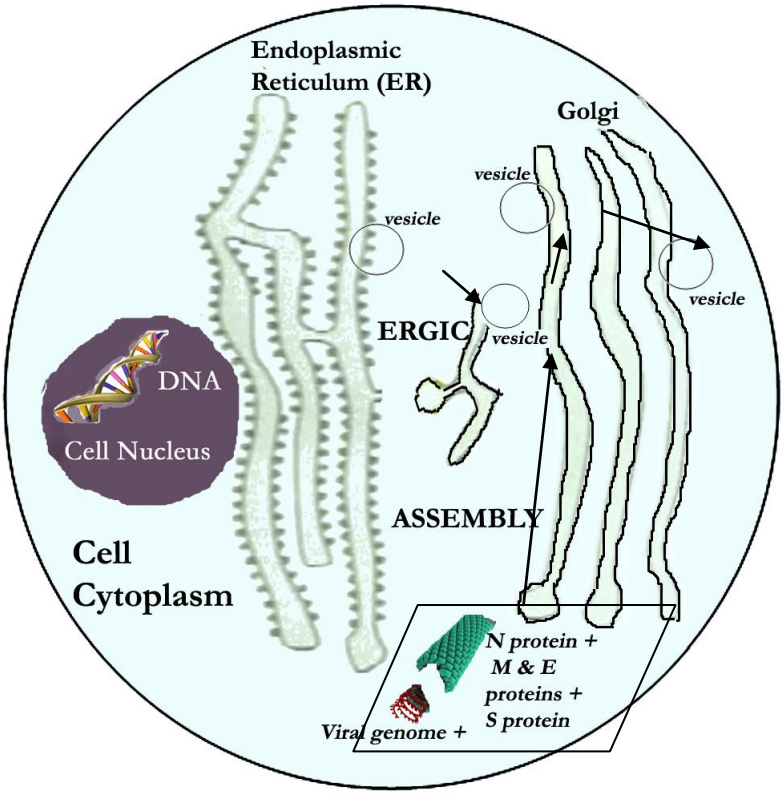
[122] Nakagawa, Lokugamage, and Makino, "Viral and Cellular mRNA Translation," 170.
[123] Lim et al., "Human Coronaviruses," 4–5; see De Haan et al., "Coronavirus Particle Assembly."

This viral assembly occurs in the *endoplasmic-reticulum–Golgi intermediate compartment* (ERGIC), briefly mentioned elsewhere in this volume in connection with intracellular trafficking. Coronavirus are unique among enveloped viruses in assembling their viral envelope at the ERGIC.[124] The illustration below, which enlarges the lower right corner of the previous one, depicts ERGIC in relation to the Endoplasmic Reticulum (ER) and Golgi-complex (GC).



The assembled virus buds through membranes in the secretory pathway from the ER to the ERGIC. *Budding*—the process by which the virus acquires its enveloping membrane—is central to assembly. The nucleocapsid (N) protein is wrapped by the membrane (M) protein, with some involvement by the envelope (E) protein.[125] The role of E remains unclear, and various proposals have been made, including closing the neck of the virus particle as the membrane is pinched off.[126]

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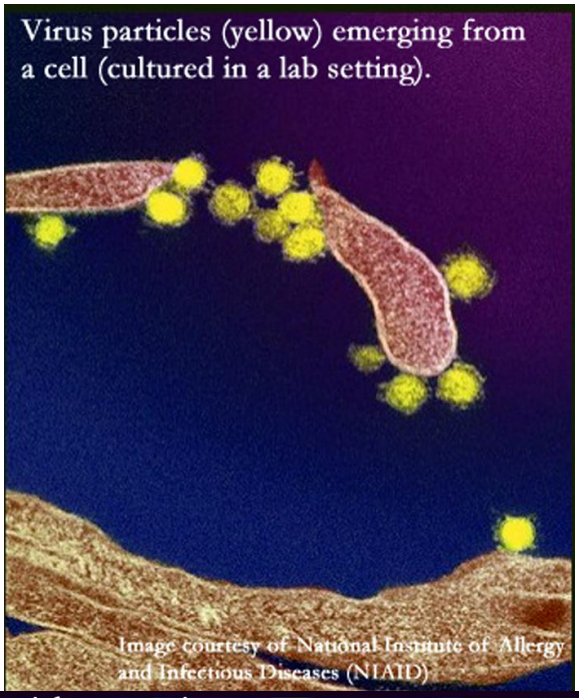
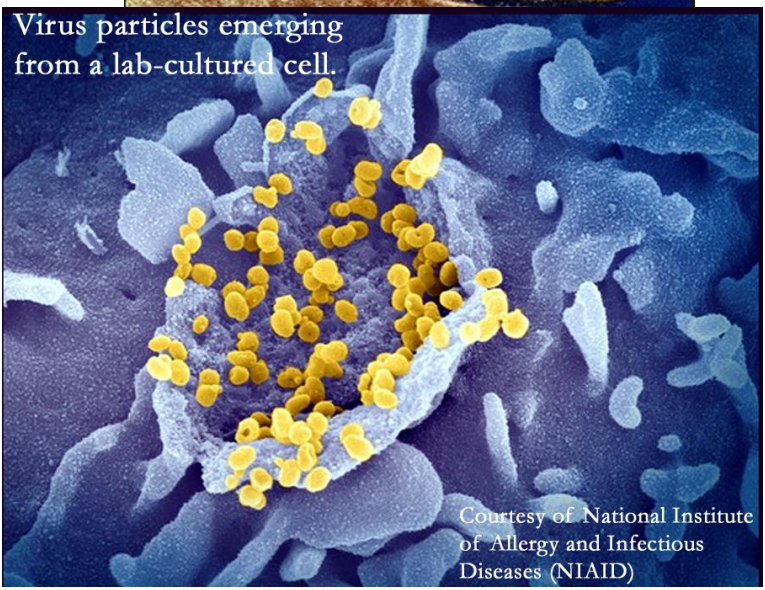
[124] Schoeman and Fielding, "Coronavirus Envelope Protein," 9.
[125] Garoff, Hewson, and Opstelten, "Virus Maturation by Budding," 1171.
[126] De Haan and Rottier, "Molecular Interactions," 181.

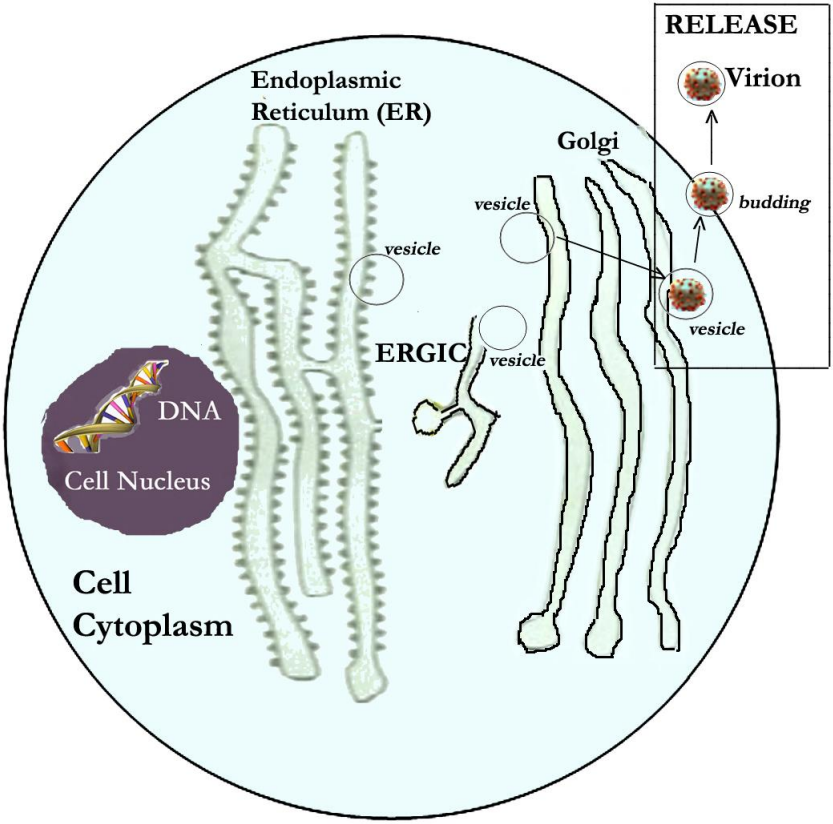
6. Release

At the host cell's outer membrane the newly assembled virus particles are released to become new virions—and infect new host cells. In the images below this final stage is shown in vivid fashion.





Because coronaviruses depend on host cell processes to complete their cycle, once they have finished with one host cell they must move on. Typically, enveloped viruses like the coronaviruses utilize the host cell's *secretory pathway*—the exit system by which proteins are secreted. This system, as shown in the illustration immediately before the above pictures, includes the endoplasmic reticulum, the Golgi apparatus, and those vesicles ('packages') shuttled between them. The Golgi complex provides part of its own membrane to form the new virus lipid envelope. Following this completion of the virus, it is packaged by the Golgi complex into vesicles and transported to the host cell's outer membrane. Through *exocytosis*, a process at the cell membrane by which packaged contents are put 'outside' (*exo-*) their 'vesicles' (*-cytosis*), the newly assembled virus particles exit the used host cell. Put formally, they "bud out as a smooth-wall vesicle to the plasma membrane to egress via exocytosis."[127]



The illustration above depicts the expected way an assembled virus particle exists the host cell to become a virion outside it. However, some variation exists.

[127] Lim et al., "Human Coronaviruses," 3.

There are two basic ways the coronavirus replicated material can be released from a host:

- *lysis*—the cell dies and its dissolution releases the viral material (*cyto-lysis*); or,
- *budding*—the cell undergoes changes caused by the virus resulting in the release of the viral material (*cytopathic effect*).

Coronaviruses employ both means, though the second of those named above—the way depicted in the illustration—is the expected, normative way.[128]

In chapter 1 we learned that *lysis* means 'dissolution' of the host cell's membrane as a means of 'letting loose' a virus. In *cytolytic release* the host cell, which is the 'vessel' (*cyto-*) of the virus 'dissolves' (*-lytic*) and thus frees the replicated viral material. This phenomena was well-observed with SARS-CoV.[129] And cytolysis matters because it, along with the magnitude of viral replication, reflect the severity of disease resulting from infection.[130] But cell death is not the norm for coronaviruses.

Cytopathic means the 'cell vessel' (*cyto-*) undergoes an 'experience' (*-pathic*) which alters it. We are more familiar with the *–pathy* part in connection to the sense of 'suffering' (e.g., *path*ology), and certainly host cells do suffer, but the broader sense is just one of an experience of change. Coronaviruses are considered *cytopathic viruses* in that they do not as a routine matter of course kill their host cells though they do alter them for their own ends.

In *cytopathic effects* (CPE) the host cell is changed by the effects wrought by the virus and the chief result is budding, in which the viral material acquires a new shell. As we have seen, budding means the viral material wraps itself in the host cell membrane material. Matured virus packaged in vesicles then employs the host cell's secretory pathways and exits by fusing with the cell membrane.[131] In this manner the virus can diffuse through the host cell's outer membrane without irrevocably damaging it or killing the cell.

While budding in the way just described is expected, there is another pathway a coronavirus might employ. Some coronaviruses (the β, or *betacoronavirus* family members) use lysosomes for exocytosis.[132] This will be considered a bit more in the next chapter. No matter the path taken, after a host cell has been used as a factory for many new viral particles it is left weakened.

Release of the newly replicated viral material completes a viral cycle. The material outside the host cell—the virion—now seeks to initiate a new cycle, repeating the steps just outlined until forcibly stopped.

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[128] Sanyai, "How SARS-CoV-2 (COVID-19) Spreads," 386, offers a cautionary observation: "Egress routes of most enveloped RNA viruses have been ill-characterized and assumed to be either via the conventional secretory pathway or by budding at the plasma membrane."
[129] Sims et al., "Severe Acute Respiratory Syndrome Coronavirus Infection."
[130] Gharamti et al., "Diagnostic Utility," 2.
[131] Tooze, Tooze, and Fuller, "Sorting of Progeny Coronavirus."
[132] Ghosh et al., "β-Coronaviruses Use Lysosomes for Egress."

Family Members

Now that we have gained an overview of shared characteristics of structure and function, and have examined the viral cycle from start to finish, we need to take time to identify individual faces in this viral family.

Tolstoy commented in his novel *Anna Karenina*, "All happy families are alike; each unhappy family is unhappy in its own way." From our human standpoint, viruses are a collection of 'unhappy families' and coronaviruses are, in particular, a source of grief. They are also distinctive among virus families. Virologist John Ziebuhr noted in 2005 that, "coronaviruses and their relatives may indeed exemplify a viral life form that, in several fundamental aspects, differs from that of other +RNA viruses."[133] It is time we step back and regain a larger picture by taking some snapshots of this viral family.

As indicated at the start of this chapter, coronaviruses are formally named as belonging to the family *Coronaviridae*, the name formed from 'corona' and the Latin *viridae*, literally, 'of the family of viruses.' So the family name labels those viruses sporting a distinctive corona. The family is enlisted in the biological order labeled *Nidovirales* (suborder *Cornidovirineae*), which includes animal RNA viruses. The *Coronaviridae* constitute the largest family in this order, of which other families are *Arterividae* (very small viral genomes, even by virus standards, causing disease in certain animals), and *Roniviridae* (a group causing disease in crustaceans). Simply for the sake of convenience we shall use the popular 'coronaviruses' in place of *Coronaviridae*.

Coronaviruses are themselves presently sorted into four groups (*genera*) based on a number of factors. These groups have many members but here we shall list only family members known to infect humans. There are seven human coronaviruses presently known:

1. *Group 1* (α, or *alphacoronaviruses*): HCoV-229E and HCoV-NL63;
2. *Group 2* (β, or *betacoronaviruses*): HCoV-HKU1, HCoV-OC43, SARS-CoV, MERS-CoV, and SARS-CoV-2; and,
3. *Group 3* (γ, or *gammacoronaviruses*): no human members (avian viruses).
4. *Group 4* (δ, or *deltacoronaviruses*): no human members (some birds, pigs, and Asian leopard cat).[134]

With the likelihood of more coronaviruses being discovered as time passes it is expected the number of identified members (and maybe groups) will grow.

Although coronaviruses infect human beings and many other animals, our interest is in those that affect humans, which means groups 1 (α) and 2 (β). In that way we have reduced our interest in the family to a few members. Four of these generally produce in people what we term the common cold; the other

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[133] Ziebuhr, "The Coronavirus Replicase," 58.

[134] See Peiris, "Coronaviruses," 587–88 (Box 1 on latter page); Helmy et al. "The COVID-19 Pandemic," 3–7 (especially Table 1, pp. 5–7), and "Coronaviridae," 435–38.

three create more serious disease. All three of these more serious coronaviruses are in group 2 (β).

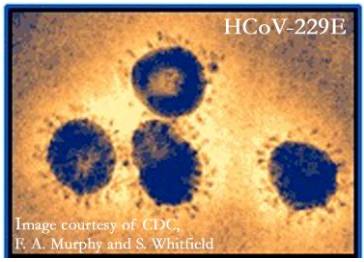
To make the situation even more transparent let us rearrange the seven into columns differentiated by symptom severity:

Less Serious	*More Serious*
HCoV-229E; HCoV-NL63; HCoV-HKU1; HCoV-OC43— all causing cold-like symptoms	MERS-CoV (causing Middle East Respiratory Syndrome); SARS-CoV (causing Severe Acute Respiratory Syndrome); SARS-CoV-2 (causing COVID-19) [135]

Obviously, those listed on the right side of this list are the ones that garner most attention, and rightly so. Therefore our attentional focus can be even further restricted, to group 2 (β), the *betacoronaviruses*.

The Less Severe Human Coronaviruses

Before we give the remainder of our attention to the three severe family members of the β group, at least some brief attention is warranted to the other human coronaviruses. The four known—and it is wise to stress *known*—coronaviruses that we can judge less severe are still the unpleasant cause of illness in humans. They are split between two family groups: HCoV-229E and HCoV-NL63 are in group 1 (α, or *alphacoronaviruses*); HCoV-HKU1 and HCoV-OC43 are in group 2 (β, or *betacoronaviruses*). All are 'seasonal,' i.e., they circulate in the human population annually. They typically result in mild symptoms of the upper respiratory tract.



The first identified human coronavirus—HCoV-229E—derives its identifier (229E) from the student specimen selected by researchers as the prototype strain of the new virus. This specimen was among those collected in the winter of 1962 from medical students enrolled at the University of Chicago. The subsequent report of this research, in 1966, suggested this be viewed as a new respiratory virus associated with mild upper respiratory illness.[136]

HCoV-NL63 (Human Coronavirus NetherLand 63)—the other member of the alpha group—is the identifying member of a closely related group (including also HCV-NL and HCoV-NH) that have been thought represent strains of the same coronavirus.[137] NL63 was first identified in an infant in 2004.[138] It usually

[135] Hasöksüz, Kiliç, and Saraç, "Coronaviruses and SARS-CoV-2," 549. Cf. Hu et al. "Characteristics of SARS-CoV-2 and COVID-19," 142–43.
[136] Hamre and Procknow, "A New Virus."
[137] Esper et al., "Coronavirus HKU1," 775.

causes just mild respiratory tract infections, though on occasion these can turn serious and even result in death. It is also associated with croup (an upper airway infection marked by a barking cough) in children. It is a kind of coronavirus that utilizes receptor-mediated endocytosis—i.e., it lets itself be 'eaten'—for entry into a host cell.[139]

HCoV-HKU1 (Human Coronavirus Hong Kong University 1[140]) is a beta-coronavirus, thus more closely associated with the more serious coronaviruses (MERS, SARS-CoV, and SARS-CoV-2), despite the finding that it is not very closely related to the other members of this branch of the family. It was first identified in early 2004 upon examination of a 71-year old Chinese man presenting with pneumonia after returning home from Shenzen, China. Subsequently it was also found in a 32-year old woman.[141] A study in 2005 in France established this coronavirus also could be found outside China. In 141 specimen samples collected during a two month period (February and March), six patients—all of them age 19 or younger (8 mos., 9 mos., 18 mos., 3 years, 5 years, and 19 yrs.)—were identified as having been infected by HKU1.[142] Another study established that it had been present, if undetected, in the United States. Patients so diagnosed had experienced upper or lower respiratory tract infection—or both.[143]

HCoV-OC43 (Human Coronavirus Organ Culture 43) was identified in a tracheal organ culture.[144] At least one study has found OC43, in comparison to the other lesser human coronaviruses, to be the most infectious (followed, in descending order, by NL63, HKU1, and 229E).[145]

Coronaviruses are estimated to account for up to 35% of the total respiratory viral activity during epidemics. In more ordinary circumstances they are estimated to account for about 15% of adult colds.[146] A large scale study in the United Kingdom of 11,663 respiratory samples collected over a three year span (2006–2009) found detection of coronaviruses across all age groups, though most common in infancy (ages 7–12 mos.; 4.86% of all cases), and overall more common in males (64.6%).[147] The less severe coronaviruses seem to display a similar symptom profile, one associated with the common cold, but both HCoV-229E and HCoV-NL63 have been implicated in studies as sometimes resulting in more severe illness, especially among children. Whether people can be infected more than once by the same coronavirus remains unanswered, especially given these viruses' penchant for mutation.[148]

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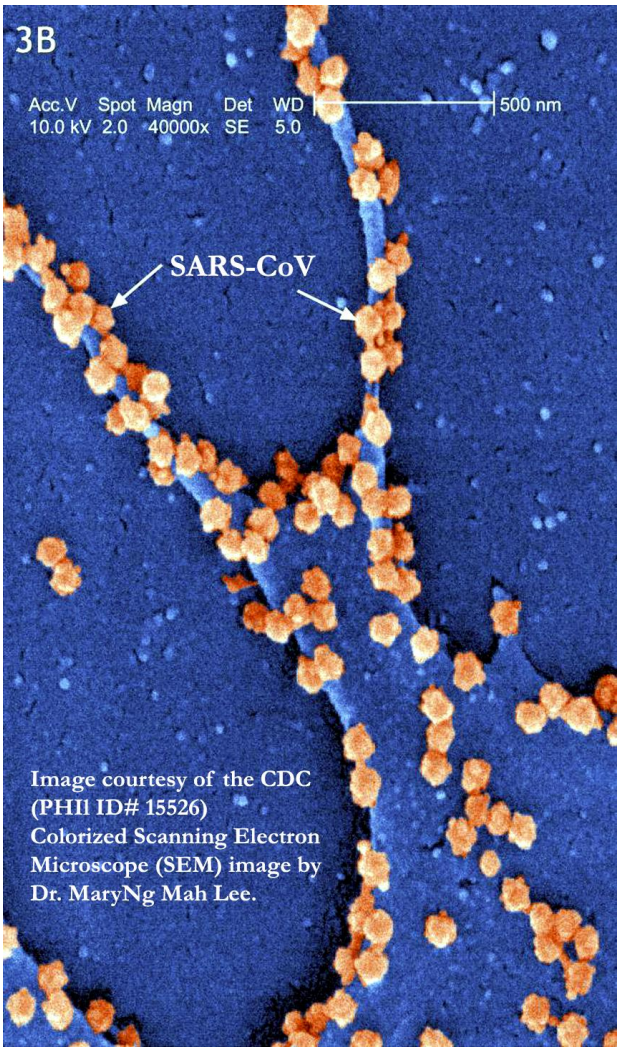
[138] Liu, Liang, and Fung, "Human Coronaviruses-229E, -OC43, -NL63, and HKU1," 429.
[139] Milewska et al., "Entry of Human Coronavirus NL63 into the Cell."
[140] Liu, Liang, and Fung, "Human Coronaviruses-229E, -OC43, -NL63, and HKU1," 428.
[141] Woo et al., "Characterization and Complete Genome Sequence of a Novel Coronavirus."
[142] Vabret et al. "Detection of the New Human Coronavirus HKU1"; see Table 1, p. 638.
[143] Esper et al., "Coronavirus HKU1 Infection in the United States."
[144] Liu, Liang, and Fung, "Human Coronaviruses-229E, -OC43, -NL63, and HKU1," 428.
[145] Dijkman et al., "The Dominance of Human Coronavirus OC43," 138.
[146] Kahn and McIntosh, "History and Recent Advances in Coronavirus Discovery," S223.
[147] Gaunt et al., "Epidemiology and Clinical Presentations of the Four Human Coronaviruses."
[148] For a review of such matters, see Williams, "A Brief History of Coronaviruses."

SARS-CoV (Severe Acute Respiratory Syndrome Corona Virus)

The coronavirus SARS-CoV, causal agent of Severe Acute Respiratory Syndrome (SARS) was the first truly deadly human coronavirus to emerge. Officially, the number of deaths attributed worldwide to SARS has not been large: at the end of the epidemic in 2003 the numbers were first reported as 8,422 people infected, with 916 deaths;[149] by 2009 these had been slightly revised to 813 deaths out of 8,437 people.[150] In either case the reported mortality rate—10.87% or 9.6%—is attention getting. So, too, is the estimated economic impact: more than $50 billion in 2009 dollars. The epidemic proved to be most prominent in China and Hong Kong, but spread to at least 29 countries.[151] However, studies indicate that SARS-CoV has a moderate mutation rate compared to other RNA viruses.[152] This is good news in terms of coping with it in the human population.



The advent of the SARS-CoV is also important for having spurred further study of the entire coronavirus family. This led to the identification of two new human coronaviruses (NL63 and HKU1).

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[149] Chan-Yeung and Xu, "SARS: Epidemiology."
[150] Holmes, *The Evolution and Emergence of RNA Viruses*, 1.
[151] Helmy et al., "The COVID-19 Pandemic," 3.
[152] Zhao et al., "Moderate Mutation Rate in the SARS Coronavirus Genome."

MERS (Middle East Respiratory Syndrome)

If SARS-CoV-2 caught attention, MERS grabbed it even more dramatically.

First termed Human Coronavirus Erasmus Medical Center (HCoV-EMC), and then variously designated, including as simply New Coronavirus (NcoV), it was finally officially named the Middle East Respiratory Syndrome (MERS) virus. It surfaced in Jeddah, Saudi Arabia in September, 2012, though it was later determined it had been present in a cluster of pneumonia cases in neighboring Jordan in April of that year.[153] Though it spread to several other countries, including the United States, the largest outbreak outside the Middle East was in South Korea (2015), where 186 cases were recorded, with 38 deaths.[154] All these latter instances could be traced back to travelers who had been in Saudi Arabia.

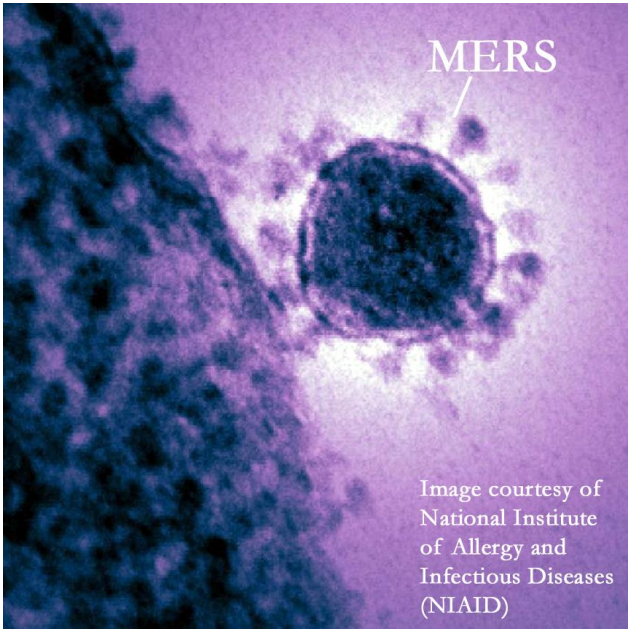

Image courtesy of National Institute of Allergy and Infectious Diseases (NIAID)

Camels have been assumed to be the intermediary species between bats and humans. The virus survives for prolonged periods in camel's milk and direct exposure to camels has been identified as a risk factor.[155]

By the beginning of 2020, MERS-CoV had been confirmed by laboratory test results in 2519 cases, with 866 deaths (34.3% fatality rate); more than 80% of these cases were in Saudi Arabia.[156]

Cases range from asymptomatic to mild to moderate to severe. In severe cases complications of severe pneumonia, acute respiratory distress syndrome (ARDS), septic shock and multiple organ failure may occur.[157]

Now that we have considered basic matters about the coronaviruses we are ready to focus on the most important member of the family—the one that has so occupied the world's attention since the winter of 2019–2020.

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[153] De Groot et al., "Commentary: Middle East Respiratory Syndrome Coronavirus."
[154] Azhar et al., "The Middle East Respiratory Syndrome (MERS)," 892.
[155] Azhar et al., "The Middle East Respiratory Syndrome (MERS)," 893–94.
[156] Helmy et al., "The COVID-19 Pandemic," 3.
[157] Azhar et al., "The Middle East Respiratory Syndrome (MERS)," 894.

Chapter 3
The Enemy: SARS-CoV-2

One of the colorful descriptors often encountered in discussions of the pandemic is that humanity is at 'war' with COVID-19. The 'enemy' is a disease that has killed millions, severely sickened millions more, and shows no signs of surrendering any time soon. To understand this enemy—this disease—we must spend some brief time focused on the virus that causes it.

The virus that produces the disease COVID-19 was first named '2019 novel coronavirus' (2019-nCoV). The word 'novel' simply means 'new'—the virus was newly discovered in 2019. The 'CoV' is shorthand for '**Co**rona**v**irus.'

The former name has given way to an 'official' name: SARS-CoV-2. The label is a short form for the name 'Severe Acute Respiratory Syndrome (SARS) Coronavirus (CoV) 2.' The first part of the name describes the *effect* of the virus: it causes a characteristic group of symptoms ('syndrome') associated with the human body's system for breathing ('respiratory') that occurs suddenly ('acute') and with great intensity ('severe'). The next part of the name describes the *biological viral family* to which the virus belongs, called coronavirus because under an electron microscope its surface shows distinctive crown-like features that produce a visual effect like looking at the sun's corona. The number '2' distinguishes it from the previously discovered SARS-CoV virus.

As noted in the previous chapter, coronaviruses are prone to mutations giving rise to *variants*. Most of these are not named. Those that are by the World Health Organization (WHO) are given names from the Greek alphabet—'alpha,' 'beta,' 'gamma,' and so forth. The originally detected strain of the virus was and remains termed the 'wild type' variant. Discussion of the variants, including the Omicron variant identified near the end of 2021, may be found at the end of this chapter.

Likenesses within the Coronavirus Family

As we also saw in the previous chapter, SARS-CoV-2 belongs to the family of viruses popularly called 'Coronaviruses' (technically named *Coronaviridae*). Within this family SARS-CoV-2 is more like some 'siblings' than others. Based on genetic nucleotide similarity, it is most closely related to two coronaviruses found in bats: CoV RaTG13 (96.3%), and SARS-like CoV ZXC21 (89%). Among human coronaviruses, as we might guess from its name, it is most like SARS-CoV (82%).[158] It belongs to a distinct group within the family that has been variously identified but is most commonly referred to as the β, or *betacoronaviruses*. This group includes five human coronaviruses (see chapter 2).

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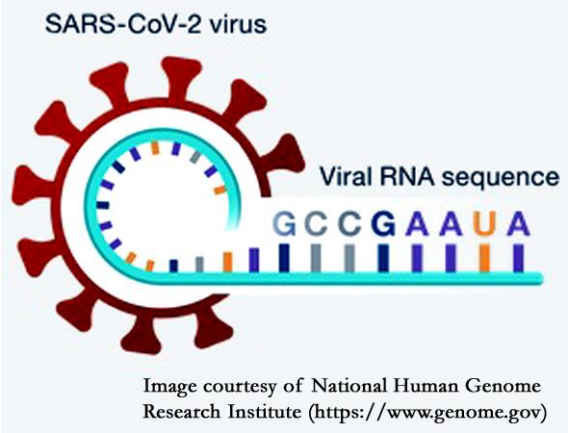
[158] Helmy et al. "The COVID-19 Pandemic," 2. Hu et al., "Characteristics of SARS-CoV-2 and COVID-19," 142, put the genome sequence identity between SARS-CoV and SARS-CoV-2 at 79%, and adds (p. 143), "SARS-CoV-2 shares more than 90% amino acid identity with SARS-CoV except for the S gene, which diverges."



The formal, technical description of SARS-CoV-2 in concise terms is: an 'enveloped positive-sense single-stranded RNA virus' (+ssRNA). Its genome, as submitted to the National Center for Biotechnology (NCBI), bears the ID NC_045512, and shows 19,903 base pair (bp) of single-stranded (ss) RNA.[159]

We saw in the previous chapter that *enveloped* means the RNA core is surrounded and protected. *Positive-sense* means the viral genome is a functional messenger RNA (mRNA)[160]—it is capable of instructing the invaded host cell what to do. *Single-stranded*, as we learned in chapter 1, differentiates RNA from double-stranded DNA, though some RNAs are also double-stranded. The *+ssRNA* is simply shorthand for positive (+), single-stranded (ss), ribonucleic acid (RNA).

SARS-CoV-2 consists of a relatively short sequence of genetic material. Within the coronavirus family SARS-CoV-2 runs on the smaller side size-wise. Various studies have determined a size in the 40–140 nm range.[161] It is only about 29,900 nucleotides (each an organic molecule made of adenine, guanine, uracil, or cytosine) that encode all of its proteins (see illustration above).

In the previous chapter we saw that all of the coronavirus family members have in their genome a *conserved order* of seven genes. This means all of them share an order of sequence for these RNA nucleic acids, and they are in an order of direction (5′ to 3′).[162] Here is that earlier illustration as a reminder.

5′	ORF1a	ORF1b	S	ORF3	E	M	N	**3′**

However, as valuable as the above is for reminding us of distinguishing genetic features shared by SARS-CoV-2 with its coronavirus kin, and for highlighting key features—three open reference frames and four structural proteins—we need to examine our viral enemy much more closely.

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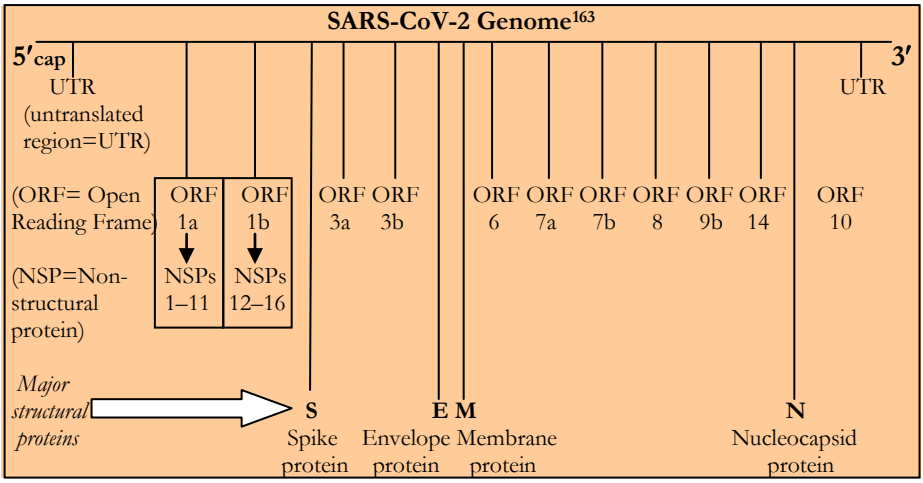
[159] Ramírez Hernández et al., "The Role of the SARS-CoV-2 S Protein," 166.

[160] Payne, *Viruses*, 98, puts it this way: "Positive or plus (+)-strand RNA viruses have genomes that are functional mRNAs."

[161] Culfari, "The Size of SARS-CoV-2 and Its Implications."

[162] See, among many others, Wang, Grunewald, and Perlman, "Coronaviruses"; also Woo et al., "Coronavirus Genomics and Bionformatics Analysis."

A more detailed genome sequence for SARS-CoV-2 looks like the following (the staggering of the components is simply to make them easier to distinguish rather than to reflect the relative length of each part):



A conveniently tight summary of the above is provided by microbiologist Zhihua Bai and colleagues: "The viral genome consists of a positive-sense, single-stranded RNA, which comprises 14 open reading frames (ORFs), encoding 16 nonstructural proteins that make up the replicase complex, nine accessory proteins (ORF), and four structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N)."[164]

Before we proceed it seems important to call attention to an underlying reality that can be easy to miss when we start discussing individual parts—*all of the above components are integrated into one viral whole*. While separating out parts for attention is useful we do so at our peril if we forget that they exist and function as integrated parts of a whole. For example, as we discuss the functioning of SARS-CoV-2 we will mention the relationship between ORFs and NSPs; the latter depend on the former. Similarly, relationships exist between ORFs and structural proteins—and among the structural proteins. Try to remember this.

In the previous, general chapter on the coronaviruses we saw, mostly in passing, some of the contributions made by a few open reading frames (ORFs) and some nonstructural proteins (NSPs). But most of our focus was on the four famous structural proteins (S, E, M, and N). Our attention will continue to be on these structural proteins and especially S for one prominent reason—these are the focus in scientific literature and provide the areas targeted in vaccine development. Things like accessory proteins remain less studied and less understood and here will be only incidentally touched upon.

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[163] Following Yadav et al., "Role of Structural and Non-Structural Proteins," 3 (Fig. 1).
[164] Bai et al., "Structure, Biological Functions, and a Potential Target," 1.

Non-Structural Proteins

Let's start with the Open Reading Frames (ORFs) and their relationship to nonstructural proteins (NSPs). As the preceding illustration indicates, ORF1 and ORF2 account for 16 of the proteins of SARS-CoV-2. This represents roughly two-thirds of the genome and the proteins associated with these ORFs are known, respectively as pp1a and pp1b proteins.[165] We saw in the previous chapter the important role these play in the life of coronaviruses.

The 16 *nonstructural* (nsp) proteins may be briefly summarized:

NSP	*Function—known or presumed (and other notes)*
nsp1	host shutoff factor: binds to mRNA entry channel; blocks host cell mRNA translation or promotes its degradation
nsp2	some regions may contribute to infectiousness (binds to prohibitin proteins)
nsp3	interacts with N protein (among other actions; differentiator from SARS)
nsp4	aids structuring of double-membrane vesicles (DMVs); possibly modulates anti-viral innate immune signaling (of RIG-1)
nsp5	main protease (M^{pro}) cleaves viral polyprotein (see chapter 2); antagonizes innate immune system
nsp6	potential transmembrane scaffold protein (as in nsp4)
nsp7	replication and transcription of viral genome (cofactor with nsp8)*
nsp8	replication and transcription of viral genome (cofactor with nsp7)*
nsp9	RNA binding protein
nsp10	aids replication fidelity (cofactor for nsp 14 and nsp16)
nsp11	(from pp1a becomes nsp12 following extension of pp1a into pp1b)
nsp12	RdRp**; assembles RNS-synthesis complex (with nsp 7 and nsp 8)
nsp13	RNA helicase; interferes with innate immune system (interferon antagonist)
nsp14	proofreading activity; adds 5′cap; interferes with innate immune system (interferon antagonist)
nsp15	evades RNA sensing; interferes with innate immune system (interferon antagonist)
nsp16	shields viral RNA from pattern-recognition receptor MDA5[166]

*Both nsp7 and nsp8 act as cofactors to bind to nsp12 ** RNA-dependent RNA polymerase

[165] Zhang et al., "Molecular Mechanism of Interaction between SARS-CoV-2 and Host Cells," 1.
[166] Table information based on Wang, Grunewald, and Perlman, "Coronaviruses," 29–30 (Table 2), and p. 6 [on nsp11]; Schubert et al. "SARS-CoV-2 Nsp1" [on nsp1]; Yan Yip et al., "Development," 5 [on nsp2]; Angeletti et al., "COVID-2019," 4 [on nsp2 and nsp3]; Davies et al., "Comparative Multiplexed Interactomics" [on nsp4]; Liu et al. "SARS-CoV-2 Nsp5," 13 [on nsp5]; Kirchdoerfer and Ward, "Structure of the SARS-CoV-2 nsp12" [on nsp 12, nsp 7 and nsp 8]; Yuen et al. "SARS-CoV-2 nsp 13, nsp14, nsp 15," 1419 [on nsp13, nsp 14, and nsp 15]. Also see Malone et al., Structures and Functions of Coronavirus Replication," 22 (Table 1).

Unfortunately, the brief summaries are plagued by their brevity, which can be misleading. Scientists are learning more and more about the nonstructural proteins as well as about the rest of what makes SARS-CoV-2 tick. So while the above table conveniently highlights a few matters it should not be regarded as complete or even fixed. For example, the table barely hints at the interrelationships among the NSPs. Two researchers, in writing about nsp12, make a telling observation:

> A number of studies have confirmed the direct interaction between nsp8 and nsp12. In addition, nsp12 has also been suggested to interact with nsp5, nsp9, and nsp13 while nsp8 has been suggested to interact with nsp7, nsp8, nsp9, nsp10, nsp13, and nsp14. The large number of interactions between nsp8 and nsp12 with the other viral nsps suggests that these two proteins form a hub for protein–protein interactions within the viral replication complex.[167]

If the illustration of the SARS-CoV-2 genome were drawn differently it would be easier to see a matter pointed out earlier in the book: between them ORF1a and ORF1b—collectively referred to as ORF 1ab—occupy about two-thirds of the genome. That illustration, simply rendered, would look like this:

The other one-third of the genome encodes four structural proteins (see next section) and accessory proteins. The difference between 'structural' and 'nonstructural' proteins is captured by the term 'structure.' A *structural protein* contributes to the organization of the whole; its functions are related to the arrangement of the virus—how it is built. This makes them especially noticeable. *Nonstructural proteins* are—by definition—not structural in nature. They are encoded by the viral genome to perform various functions, notably those related to viral replication.

Before we turn our attention to the structural proteins, let's briefly talk about the *accessory proteins*, to date the least understood and studied of the viral proteins. Some eleven accessory proteins have been identified (ORF3a, ORF3b, ORF3c, ORF3d, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF9c and ORF10). Some are implicated in viral replication processes, but most seem inessential for that central task. They do appear to be involved in different mechanisms of *pathogenesis*—the development of disease.[168]

The total number of proteins identified for SARS-CoV-2 has grown to 31. These include 16 nonstructural, 11 accessory, and 4 structural. The last named are the most famous and the focus of our next section.

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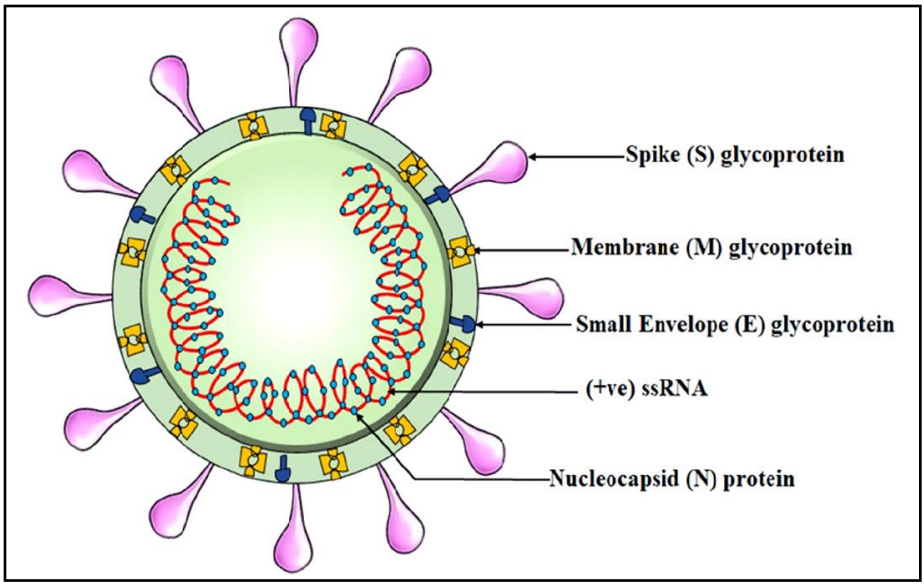
[167] Kirchdoerfer and Ward, "Structure of the SARS-CoV-2 nsp12," 5.

[168] Redondo et al., "SARS-CoV-2 Accessory Proteins," 2; cf. Ramírez Hernández et al., "The Role of the SARS-CoV-2 S Protein," 166, who list ORFs 3a, 3b, 6, 7a, 7b, 8a, 8b, 8c, 9b, and 14.

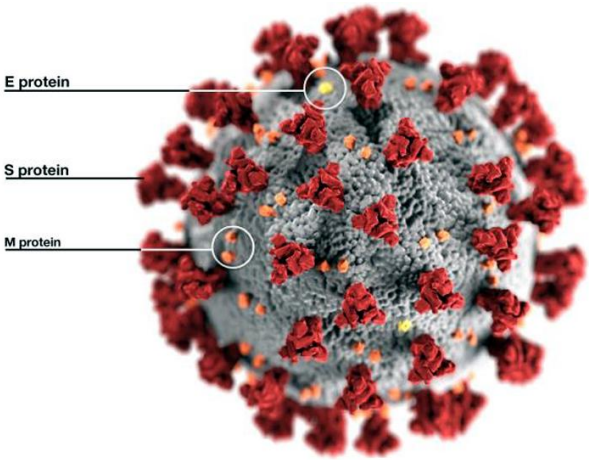
The Four Major Structural Proteins

The illustration below provides an overview of the well-known structural parts of SARS-CoV-2—the RNA core, nucleocapsid, and envelope—parts which have four structural proteins (S, M, E, and N).



The above illustration is from Vibhuti Kumar Shah, Priyanka Firmal, Aftab Alam, Dipyaman Ganguly, and Samit Chattopadhyay. "Overview of Immune Response During SARS-CoV-2 Infection: Lessons from the Past." *Frontiers in Immunology* 11 (Aug., 2020) art. 1949. doi: 10.3389/ fimmu.2020.01949. Material is from page 3 and is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/ legalcode). Only the image in part A of Figure 1 has been retained; it has not been altered.

The Center for Disease Control (CDC) has prepared the much more familiar illustration of the virus, found at the left. It highlights three of the four structural proteins.



The illustration at left is courtesy of the CDC Public Health Image Library (PHIL), at https://phil.cdc.gov). The content providers are Alissa Eckert, MSMI, and Dan Higgens, MAMS. The image is of ultrastructural morphology exhibited by coronaviruses.

Throughout this volume we have seen the attention paid to proteins when it comes to understanding viruses. This certainly holds true for studying SARS-CoV-2.[169] It is especially true for the structural proteins. These are:

- *Spike protein* (S)—found on the outer viral membrane it provides a way to attach to host cells.
- *Envelope protein* (E)—an infrequent but important presence on the outermost part of the virus.
- *Membrane protein* (M)—the chief protein making up the outer surface of the virus.
- *Nucleocapsid protein* (N)—the protein that binds to the inner RNA genomic core and provides it a protective shell.

Our review here will focus on matters particular to SARS-CoV-2. Directly below are some quick notes of focus. The more general discussion of each is found in the previous chapter. As we examine the viral cycle the role of these will be further explained.

The Spike (S) Protein

The *S (Spike) protein* is the distinctive spike which dots the virus' surface and identifies it as a 'corona'-virus. By way of quick review from the previous chapter, here is the S protein structure:

N-terminal domain (NTD)	*transmembrane domain* (TMD)	*C-terminal domain* (CTD)

At the N-terminus is a signal peptide, followed by the S1 subunit, and the S2 subunit. The NTD extrudes from the cell (i.e., is 'extracellular'). The TMD is anchored in the membrane itself. The CTD is both short and intracellular.[170]

Spike protein is used to attach to a host cell. In brief, in the simplest terms, S protein is:

- a stalk with an outer cap,
- where the cap is used for attachment to a host cell,
- by means of subtle trickery.

This protein is prominently involved in infection of a host and that involvement is described more completely later. Because of its key role in attachment to host cells it has been an obvious target of vaccines. However, as we shall see when examining variants of SARS-CoV-2 at the end of this chapter, mutations of the spike protein constitute a constant and troublesome issue.

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[169] For brief descriptions of all four structural proteins, see Schoeman and Fielding, "Coronavirus Envelope Protein," 2. For detailed descripton of the virus' transcriptome, see Kim et al., "The Architecture of SARS-CoV-2 Transcriptome."

[170] Huang et al., "Structural and Functional Properties," 1141–42.

The Envelope (E) Protein

The *E (Envelope) protein*—the smallest and least understood of the structural proteins—is named for its function of contributing to the creation of an outer wrapping (the 'envelope') for the virus. SARS-CoV shares 91% sequence identity with SARS-CoV-2 for this protein.[171] Compared to both S protein and M protein, E protein is highly conserved and stable. An examination of 797 genomes of the SARS-CoV-2 virus found only 3 variants of the E protein.[172]

Researcher Rohitash Yadav and colleagues describe it as follows: "The E protein topology of SARS-CoV-2 represented a five-helix bundle surrounding a dehydrated narrow pore with bipartite channel."[173] As we saw in the previous chapter the E protein has three basic domains:

N-terminal domain (NTD)	*transmembrane domain* (TMD)	*C-terminal domain* (CTD)

But while this may tell us what it *looks* like, it doesn't tell us what it *does*. Despite having a relatively low number of copies per viral particle, the E protein appears to be involved in a number of functions related to different phases of viral infection.[174] One stage of the viral cycle in which it plays an important mediating role is viral budding and release from the host cell.[175]

The Membrane (M) Protein

The *M (Membrane) glycoprotein* is perhaps most noted for its role in defining the shape of the envelope formed for SARS-CoV-2. It is the most abundant protein and works with E, the least abundant structural protein, in forming this viral envelope. But M protein has important interactions with the other structural proteins, too. The interaction of M protein with the spike (S) protein is a necessary one for retention of S protein in the ER-Golgi intermediate compartment (ERGIC) and its integration into new viral particles during the assembly phase of the viral cycle.[176] It also is thought to stabilize the N protein and promote completion of virus assembly inside the host cell.[177]

The M protein, as we saw last chapter, has three domains:

N-terminal ectodomain	*triple spanning transmembrane domain*	*C-terminal endodomain*

'Ecto' means 'outside' the virus; 'endo' means 'within' it.

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[171] Bianchi et al., "SARS-CoV-2 Envelope," 2; cf. Tilocca et al., "Immunoinformatic Analysis," 184.
[172] Bianchi et al., "SARS-CoV-2 Envelope and Membrane Proteins," 2.
[173] Yadav et al., "Role of Structural and Non-Structural Proteins," 4.
[174] Tilocca et al., "Immunoinformatic Analysis," 184.
[175] Mandala et al., "Structure and Drug Binding of the SARS-CoV-2 Envelope Protein," 1203.
[176] Alharbi and Alfrefaei, "Comparison of the SARS-CoV-2 (2019 n-Cov) M Protein," 1.
[177] Jörrißen et al., "Antibody Response to SARS-CoV-2 Membrane Protein," 2.

The SARS-CoV-2 M protein has 90% homology with the M protein of SARS-CoV. In structure it has a triple helix bundle and, as represented in the domain sketch above, forms a single 3-transmembrane domain (i.e., it spans the membrane bilayer three times). It leaves a short NH_2 terminal domain outside the virus shell and a long COOH terminus (the cytoplasmic domain) inside the virion.[178] This structure may involve it in functions related to rapid proliferation of the virus, viral replication, and evasion of the host immune system.[179]

Membrane protein is also noted for its greater stability than spike protein. An examination of 797 genomes for SARS-CoV-2 found only seven mutations.[180] This more conserved nature of M protein has made it a desirable target for prevention (vaccine) and treatment considerations.

The Nucleocapsid (N) Protein

The *N (Nucleocapsid) protein* comprised of 419 amino acids is encoded by the ninth Open Reading Frame (ORF 9).[181] As we learned in the last chapter, N binds with the viral RNA and covers it. The N protein is essential for the virus' replication and a part of the viral life cycle known as 'genome packaging,' whereby the replicated genome of the virus is placed into a protective envelope.[182] As we also saw in the previous chapter, a large portion of this protein—the Linking (LKR) region between the *N-terminal domain* (NTD) and the *C-terminal domain* (CTD)—is intrinsically disordered.

The N protein of SARS-CoV-2 shares substantial likeness with other coronavirus N proteins. In discussing this protein before, we distinguished the three domains listed above. Now we need to expand that a bit. The N protein can be divided into five parts, but exactly how these are represented sometimes differs. We need not for our purposes examine more than one representation:

N-arm	N-terminal domain (NTD)	Linking region (LKR)	C-Terminal domain (CTD)	C-tail[183]

Three of these are intrinsically disordered regions (IDRs): the N-arm, the C-tail, and the LKR. However, all five domains have been predicted to bind RNA.[184]

During virion production it assembles into viral *RNA-protein complexes* (RNPs) to package the viral RNA. It also interacts with the M protein to recruit the viral genome.[185]

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[178] Mousavizadeh and Ghasemi, "Genotype and Phenotype of COVID-19," 160.
[179] Thomas, "The Structure of the Membrane Protein," 343.
[180] Bianchi et al., "SARS-CoV-2 Envelope and Membrane Proteins," 2.
[181] Bai et al., "Structure, Biological Functions, and a Potential Target," 2.
[182] Cubuk et al. "The SARS-CoV-2 Nucleocapsid Protein."
[183] Bai et al., "Structure, Biological Functions, and a Potential Target," 2.
[184] Cubuk et al. "The SARS-CoV-2 Nucleocapsid Protein," 2; Ye, Lu, and Corbett, "Structural Basis for SARS-CoV-2 Nucleocapsid Protein," 2.
[185] Ye, Lu, and Corbett, "Structural Basis for SARS-CoV-2 Nucleocapsid Protein," 2.

How SARS-CoV-2 Infects Human Host Cells: The Replication Cycle

SARS-CoV-2, as a 'positive-sense' RNA, can act as mRNA, meaning it can send the very specific message to an infected cell to make the proteins the virus desires (i.e., virus proteins) rather than what the host's DNA intends. Using the language of metaphor, it subverts the cell's intended work orders. Instead of carrying out its normal functions the host cell is turned into a factory for replicating and sending out the virus.[186]

By way of reminder, here again are the six distinct stages of the viral cycle.

1. *Attachment*—the virus must come into contact with a host cell.
2. *Penetration and Fusion*—the virus must enter the host cell and fuse.
3. *Fusion and Uncoating*—once inside, the virus coating over the genomic RNA must be removed.
4. *Replication*—the RNA issues instructions to reproduce itself.
5. *Assembly*—newly produced viral material is packaged.
6. *Release*—the newly packaged virus replicates are set loose outside the host cell to become virions ready to infect other host cells.

These six steps are artificially distinguished; the process is more seamless than the divisions may suggest. The illustration below shows the 'flow' of the cycle.

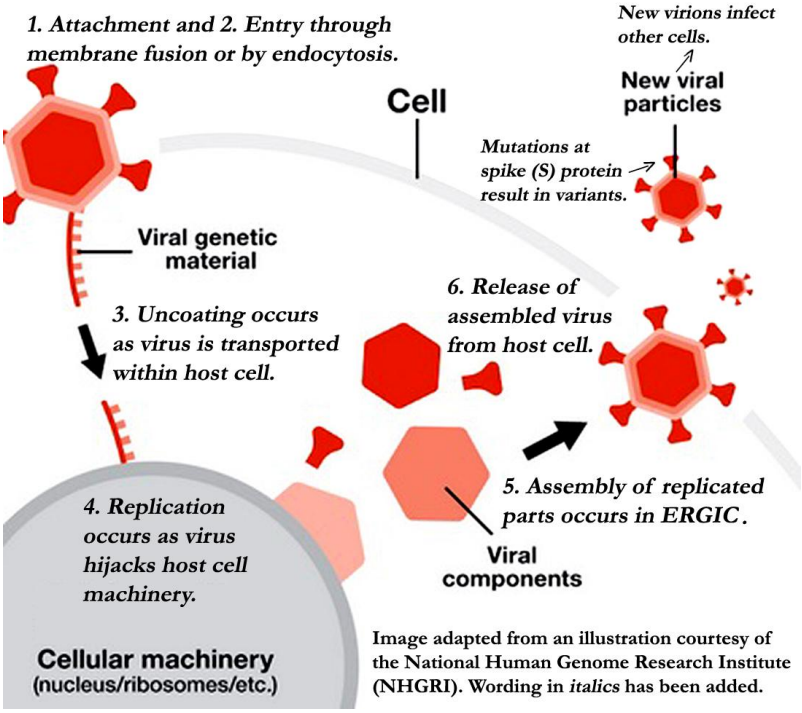


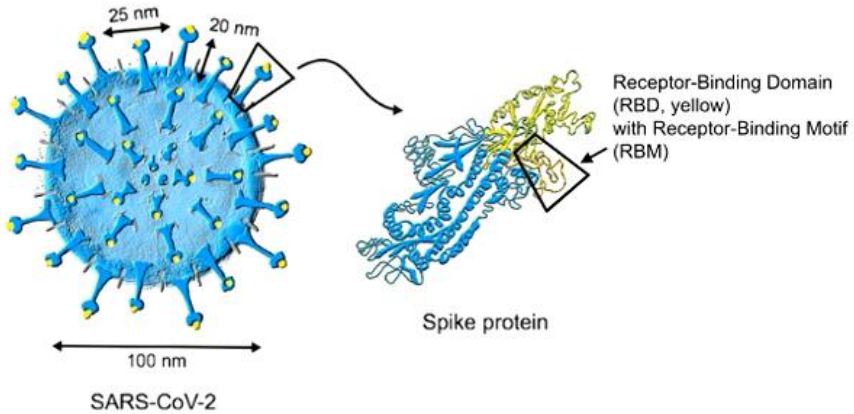
Image adapted from an illustration courtesy of the National Human Genome Research Institute (NHGRI). Wording in *italics* has been added.

[186] For the science of this process, see Sanyal, "How SARS-CoV-2 (COVID-19) Spreads," and Mattheson and Lerner, "How Does SARS-CoV-2 Cause COVID-19?"

1–2. Attachment and Penetration with Fusion

Anyone who has even a nodding acquaintance with COVID-19 has heard of the famous part of the virus that causes it—the spike (S) protein. It sits on the surface envelope of SARS-CoV-2 and does the dirty work of initiating exploitation of a host cell. It is eminently suited for its work. The S protein is both flexible and highly mobile.[187] Each spike can bind to several receptors on the host cell—an ability facilitating receptor clustering and thereby viral entry.[188]

The S protein consists of 'spikes' at the top (S1 subunit) and a 'stem' beneath (S2 subunit). The S1 subunit at the top has a region called the receptor-binding domain (RBD). An area of the RBD is known as the *receptor-binding motif* (RBM), and it is the functionally most significant part of the RBD. It can be further distinguished as having two regions (simply labeled 'region 1' and 'region 2') that become the interface of the virus with the surface receptors of the host cell.[189] The illustration below highlights this region.



Structure of SARS-CoV-2. Coronaviruses have their names from the typical spikes which are made of the spike (S) protein that is inserted in the lipid bilayer membrane of the virus. The receptor-binding domain (RBD) and its receptor-binding motif (RBM) enable interaction with the cell surface receptor ACE2 mediating entry of the virus into host cells.

Illustration courtesy of Martin F. Bachmann, Mona O. Mohsen, Lisha Zha, Monique Vogel and Daniel E. Speiser, "SARS-CoV-2 Structural Features May Explain Limited Neutralizing-Antibody Responses." *NPJ Vaccines* 6/2 (Jan. 24, 2021). https://doi.org.10.1038/s41541-020-00264-6. This is Fig. 1, p. 2, of the article, and it is made available by Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0). The illustration has not been changed but the Figure 1 description has been changed as follows: the label 'Fig. 1' has been deleted as has the descriptive wording that follows what is above.

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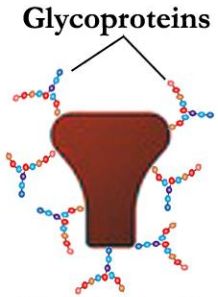
[187] Römer, Römer, and Wallis, "Flexibility and Mobility." They note (p. 3) a particularly high mobility in the S^B part of the Spike protein S1 subunit.
[188] Knyazev, Nersisyan, and Tonevitsky, "Endocytosis," 3.
[189] Yi et al., "Key Residues of the Receptor Binding Motif," 621.

1a. Attachment: General Description

SARS-CoV-2 uses a two-step process to enter a human host cell. First, the virus uses its S (Spike) proteins to attach to a specific place on a cell (a receptor site). The RBD region of the S1 subunit in its natural state lies flat and is thus disguised. Not content to stay that way at all times, it flexes, alternating between this at-rest position and one ready for action. In the 'down' position the RBD is closed; in the 'up' position the RBD is open.[190] Before it fuses with a host cell—i.e., while in its prefusion state—the S protein can seem 'closed for business.' To bind successfully the S protein's RBD has to be in a standing upright position. But that position can flag it to the immune system. It bears repeating that *the S protein varies between this standing-up position for binding, and then lying down to evade detection*. Combined with its manner of cell fusion to inject its own genome after successful attachment, SARS-CoV-2 is able to gain rapid entry, reproduction, and spread—all while evading too early detection.[191]

Upon interacting with a host cell the S protein undergoes extensive structural rearrangement. These changes are what make fusion with the host cell possible. Spike proteins are aided by a particularly effective feature—they are covered with complex sugars (*glycans* called *glycoproteins*) that camouflage the virus, thus evading immune system detection.[192] SARS-CoV-2 uses its highly glycosylated spike glycoproteins to bind to the host cell receptors.[193] Glycan is important to other parts of the virus too; the viral envelope protein (E) is regularly modified by the addition of complex glycan structures that represent half of the molecular weight.[194]



Glycoproteins provide a sugary coat of disguise.

Remember, before fusion can occur the two subunits of the S protein must be detached from one another through the process of cleavage by enzymes called proteases. As explained in the preceding chapter, cleavage of the S protein is variable in coronaviruses. SARS-CoV-2, like the extremely deadly MERS, has cleavage during biosynthesis in the infected cell.[195] Part of what makes SARS-CoV-2 so infectious is that it has multiple cleavage sites, increasing the probability that such will occur.

1b. Attachment: The Role of Specific Receptors

Attachment requires something to latch onto—and what SARS-CoV-2 latches onto through the S protein is called a 'receptor' because it is the host

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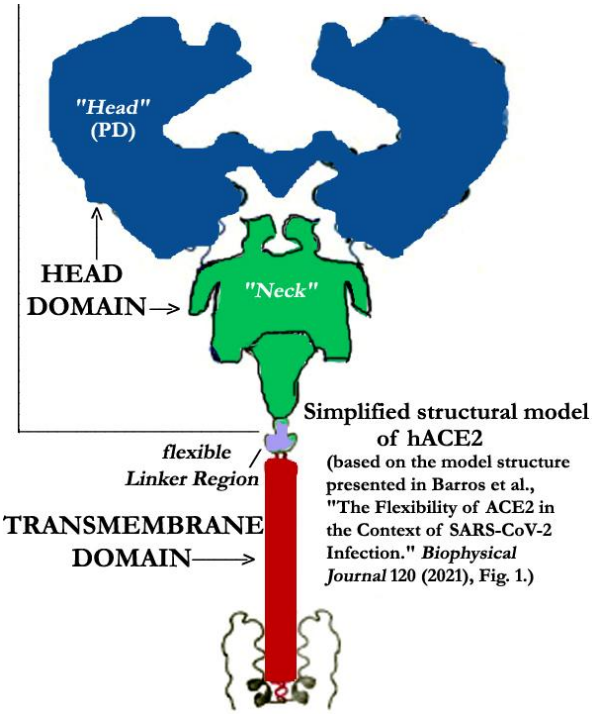
[190] Juraszek et al., "Stabilizing the Closed SARS-CoV-2 Spike Trimer," 2; also Shang et al., "Cell Entry Mechanisms of SARS-CoV-2," 11727.
[191] See Shang et al., "Cell Entry Mechanisms."
[192] Huang et al., "Role of Structural and Non-Structural Proteins," 1141.
[193] Petrović, Lauc, and Trbojević-Akmačić, "The Importance of Glycosylation."
[194] Ramírez Hernández et al., "The Role of the SARS-CoV-2 S Protein," 166.
[195] Jackson et al., "Mechanisms of SARS-CoV-2 Entry into Cells," 1.

cell's way of 'receiving' from outside itself. As indicated in the last chapter, the S protein chemically recognizes a very specific kind of receptor, labeled *hACE2* (human angiotensin-converting enzyme 2). It is time to look more closely at this receptor. *Where* the virus enters the host to do its work helps explain *why* certain parts of the body are severely affected. The hACE2 receptor plays a key role in regulating blood pressure and it is found in many places in the human body, most notably the epithelial cells of the respiratory system. But hACE2 sites also are found in the cardiovascular system, gastrointestinal tract, and in the kidneys, liver, and nervous system. Impairment of hACE2 in the cardiovascular system, for example, can lead to severe cardiac dysfunction. Similar ill effects can be found in the other places just mentioned when hACE2 function is compromised.[196]

Broadly characterized, the hACE2 receptor has two domains each with specific functions:

N-terminal peptidase domain (PD)	*C-terminal collectrin-like domain* (CLD)
↓	↓
recognizes RBD	mediates homo-dimerization

The hACE2 receptor is a *homodimer*—a two part macromolecule made of joined identical monomers—with both 'open' and 'closed' structures. These dimers can accommodate two viral spike proteins through each ACE monomer. The CLD joins the identical subunits to form a single compound (*homo-dimerization*). The PD of hACE2 recognizes the viral receptor-binding domain (RBD), facilitating attachment of the virus to the host cell.[197]



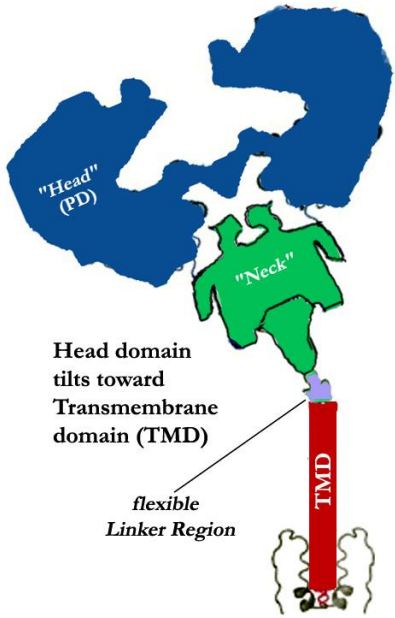
Simplified structural model of hACE2
(based on the model structure presented in Barros et al., "The Flexibility of ACE2 in the Context of SARS-CoV-2 Infection." *Biophysical Journal* 120 (2021), Fig. 1.)

The structural profile of ACE2 has been mod-

[196] Salamanna et al., "Body Localization of ACE-2," 3. Also see Figure 1 (p. 4). See the entire article for specification of effects in different organs. Also see Wang, Grunewald, and Perlman, "Coronaviruses," 6.

[197] Wu et al., "Advances in Research on ACE2," and Barros et al., "The Flexibility of ACE2."

eled by researchers and a very simplified representation of that model looks like the preceding image. The 'head,' or PD region binds to the RBD of SARS-CoV-2. The smaller 'neck' region is where most of ACE2's homodimer interactions transpire.[198]



The 'head' on the 'neck' seems to be flexible; simulations show it bends the 'head' toward the membrane. As one monomer moves thus the other acts in concert so that the two 'head' monomers remain at relatively the same distance from each other (at left).[199]

The viral RBD seems not to be bothered by this flexibility, remaining relatively stable, but it serves to divide the RBD/ACE2 interface into three interacting regions. This S1/ACE2 complex may take advantage of ACE2's flexibility and use the tilting as a mechanical force bringing the virus and cell membrane closer, leading to fusion. The flexibility may also be what allows ACE2 receptor dimers to bind with a single spike protein having two or more of its RBDs in the 'up' position. It may be, in fact, that this flexibility can allow three ACE2s per S protein—an advantage to the virus in infection.[200]

As important as the hACE2 receptor is, there are other players involved. That is because the binding of the S protein to the host cell seems to occur at a place distant from the hACE2 enzyme pocket where it might be expected. Thus, hACE2 seems not to be a direct S-activating protease (i.e., catalyzing enzyme).[201] That means *cofactors* should be anticipated—and sure enough, some have been implicated. These include:

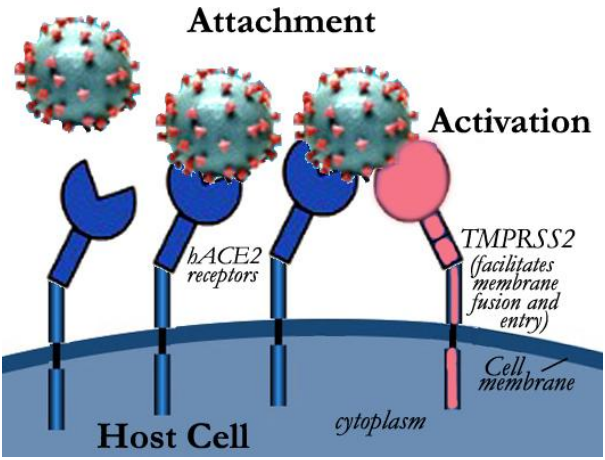
- TMPRSS2, and
- neuropilin-1.

The enzyme named *transmembrane serine protease 2* (TMPRSS2) is abundantly expressed in lung tissue and elsewhere. It mediates cleavage of hACE2, priming and activating fusion of the virus with the host cell membrane. In a number of the human coronaviruses (HCoV-229E, SARS-CoV, MERS) TMPRESS2 has been established as the major host cell protease enabling viral entry. This also

[198] Barros et al., "The Flexibility of ACE2," 1072.
[199] Barros et al., "The Flexibility of ACE2," 1075–76.
[200] Barros et al., "The Flexibility of ACE2," 1078–81.
[201] Rahman et al., "Virtual Screening of Natural Products," 2.

appears to be the case with SARS-CoV-2, but a complicating factor may be that variants of TMPRESS2 affect the degree of infection by SARS-CoV-2.[202]

TMPRSS2 also has been implicated in the widely noticed disparity between males and females in the experience of COVID-19. TMPRSS2 expression is regulated by *androgen*—a masculizing hormone—in certain cells in the lungs; differences in androgen levels thereby influence differences in disease experience.[203]



TMPRSS2 primes S2 of the spike protein, but the protein *Neuropilin-1* (NRP1) binds directly to the furin-cleaved S1 fragment of the viral S protein. While this seems not to affect SARS-CoV-2 attachment at ACE2, it does seem to promote entry and infection by the virus.[204] NRP1, a transmembrane glycoprotein, is a cell-surface receptor expressed by endothelial cells. Put simply, this is a receptor on cells forming the membrane that lines the inside of the heart and blood vessels. As heart specialists Peter Libby and Thomas Lüscher put it in the title of a 2020 journal article, "COVID-19 is, in the end, an endothelial disease." They explain that endothelial cells are crucial in host defense against pathogens because they can, through their receptors, sense danger and send warning signals.[205]

NRP1 facilitates SARS-CoV-2 cell entry—and thus infectivity—by becoming involved in virus-host cell interactions, especially in those places where NRP1 is abundant (e.g., pulmonary and olfactory cells). The virus' S1-S2 junction is one accessible to receptor interactions—and cleavage at this site provides the kind of furin-cleaved substrate that NRP1 requires for its effects. It appears that when NRP1 is coexpressed with hACE2 and TMPRSS2 markedly enhanced infection results.[206]

NRP1 has been implicated in the neurologic features of COVID-19. It facilitates entry of SARS-CoV-2 into the central nervous system (CNS) and is highly expressed in some areas of the brain (e.g., hippocampal formation).[207]

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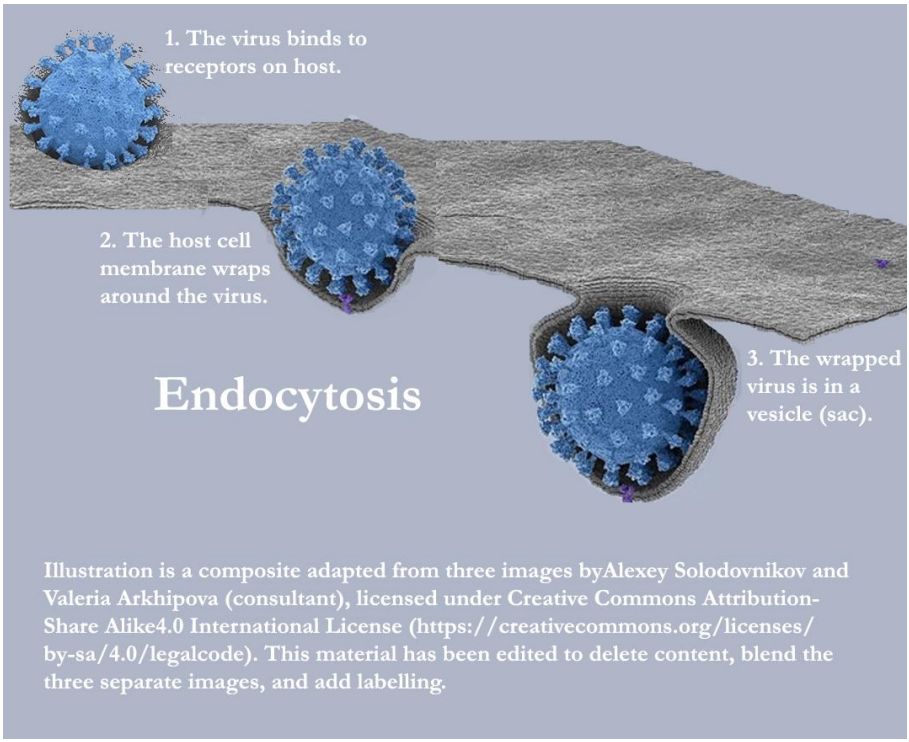
[202] Schönfelder et al., "Transmembrane Serine Protease 2."
[203] Qiao et al., "Targeting Transcriptional Regulation of SARS-CoV-2," 10. Also see Li et al., "Distinct Mechanisms for TMPRSS2 Expression."
[204] Daly et al., "Neuropilin-1 Is a Host Factor."
[205] Libby and Lüscher, "COVID-19 Is, in the End, an Endothelial Disease."
[206] Cantuti-Castelvetri et al., "Neuropilin-1 Facilitates SARS-CoV-2 Cell Entry," 856.
[207] Davies et al., "Neuropilin-1 as a New Potential SARS-CoV-2 Infection Mediator."

1–2. Attachment and Penetration via Receptor-Mediated Endocytosis

A second way of entry is through *receptor-mediated endocytosis*. It is listed second here because SARS-CoV-2 appears to prefer the previously described means of attachment and entry by fusion.[208] Researchers Armin Bayati and colleagues observe, "Endosomal entry mechanisms provide many advantages to the viruses by allowing them to efficiently spread while evading host immune surveillance."[209]



The general process of endocytosis for coronaviruses was described and illustrated in the previous chapter. Cell entry is via host receptors (hACE2) that absorb the virus through an inward budding pouch (technically termed 'invagination') formed from the cell membrane that then dissolves within the host cell. It is this later process that moves the virus from attachment and entry into the next phase of uncoating. Metaphorically, the virus finds an 'open mouth' into the cell (step 1: via receptors), then lets itself be 'eaten' (step 2: endocytosis).[210] This process, in the manner specific to SARS-CoV-2, is what must now be described.

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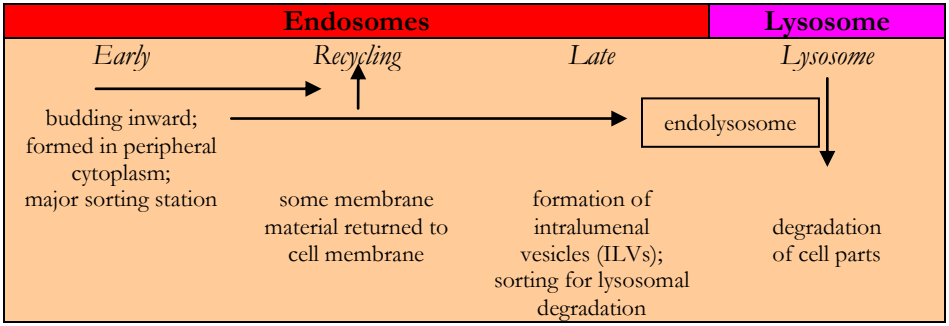
[208] Jackson et al., "Mechanisms of SARS-CoV-2 Entry into Cells," 12. For a composite illustration of the fusion and endocytosis means of entry, see Figure 3 (p. 11) in the same article.
[209] Bayati et al., "SARS-CoV-2 Infects Cells," 9.
[210] Huang et al., "Structural and Functional Properties," 1144.

For SARS-CoV-2 this endocytosis is *mediated*, meaning that more than simply the virus-receptor players are involved. Although the mechanisms of endocytosis remain unclear,[211] two substances merit recognition:

- *Clathrin*—a protein used near the start of endocytosis and,
- *Cathepsin L* (CatL)—a protein used later in endocytosis.

Although both are proteins, they serve very different functions. Clathrin serves the cell transport system by providing a covering ('coat') as a vesicle ('package') is made to carry SARS-CoV-2 into the host cell. Cathepsin L acts later on as a protease, serving as an enzyme to initiate important changes in the viral material. Both 'mediate' what happens, and both are sometimes named for this process of endocytosis—'clathrin-mediated endocytosis' or 'cathepsin L-mediated endocytosis.' But it is important not to see these kinds of labels as speaking of two completely independent types of cell entry. They are best used as drawing focus to one or another part of a general process.

These two players are part of the same dance and must be viewed as such, even though strictly speaking they are never partners with each other. Instead, they take turns dancing with SARS-CoV-2. To understand the complex dance that is endocytosis we need to speak again of the transport system within the host cell. Our focus here is on the *endosomal-lysosomal system*, an intracellular set of membrane-enclosed compartments (tubulo-vesicular structures).[212] This system can be depicted as a series of compartments like this:



The arrows convey the sense of movement. As typically described, as the SARS-CoV-2 package is formed it enters into the endosomal-lysosomal system for transport within the cell—just as would be any healthy substance the cell ingested for its own nourishment. In this 'digestive process,' as we might call it, important changes occur—and these involve both clathrin and cathepsin L.

Thus, to the above overview of the endosomal-lysosomal system we can now add a different illustration of endocytosis to highlight the roles of clathrin (early) and cathepsin L (later).

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[211] Bayati et al., "SARS-CoV-2 Infects Cells," 2, 6.

[212] My discussion here follows Hu et al., "The Endosomal-Lysosomal System."

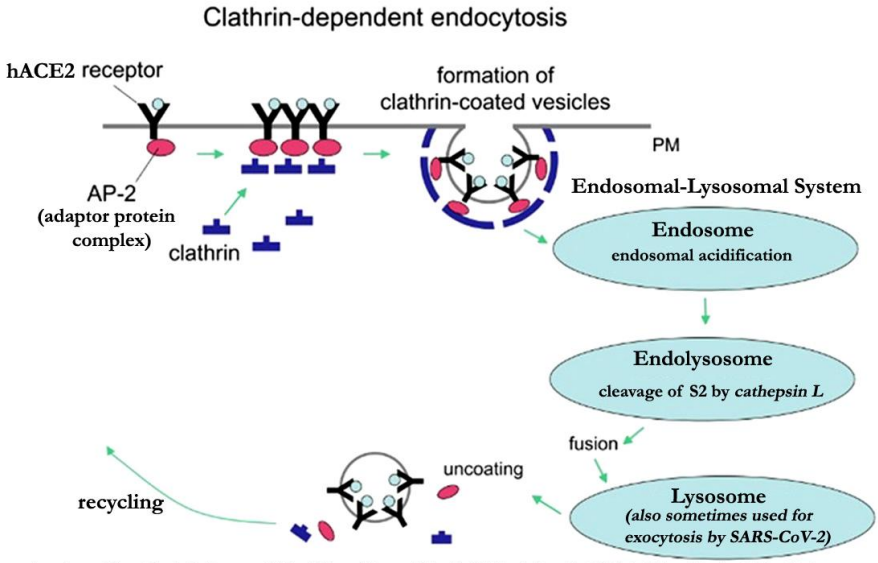

Illustration adapted from Barth D. Grant and Miyuki Sato, "Intracellular Trafficking" (Jan. 21, 2006) in *WormWood*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.77.1, http://www.wormbook.org. Made available by Creative Commons Attribution (CC-By-2.5) license (https://creativecommons/org/licenses/by/2.5legalcode). The original figure has been modified by extensive alterations. The drawn images have been retained but rearranged below the top portion of the illustration. The one original blue oval has become three--all with my labelling. Clarifying text has been added (hACE2, adaptor-protein complex, Endosomal-Lysosomal System, etc.).

In *clathrin-mediated endocytosis* clathrin works with hACE2.[213] SARS-CoV-2 is internalized by the host cell at clathrin-coated pits that bud into packages ('vesicles') formed from the cell membrane.[214] Clathrin serves as the coat for the vesicle, as shown in the above illustration.

In *cathepsin L-mediated endocytosis*, after hACE2-mediated entry into the host cell, and after mediation by clathrin as explained above, the SARS-CoV-2 material interacts with cathepsin L (a member of the cysteine class of cathepsins). The S2 subunit site is cleaved by cathepsins in the late endolysosome (a compartment which is the product of fusion of an endosome and lysosome). The mediation of cathepsin L releases the fusion peptide that initiates fusion pore formation.[215]

All three of the viral membrane proteins (S, M, and E) are initially inserted into the host. Regardless of how they enter—whether through fusion or endocytosis—the virus begins to change after contact with the host receptors. Once internalized these changes continue. The structure of SARS-CoV-2, and hence its structural proteins, must undergo deconstruction as the virus passes through the interior of the cell. This deconstruction permits the viral RNA to engage the host cell and take command. The process of attachment leads seamlessly to that of entry and, then, to uncoating.

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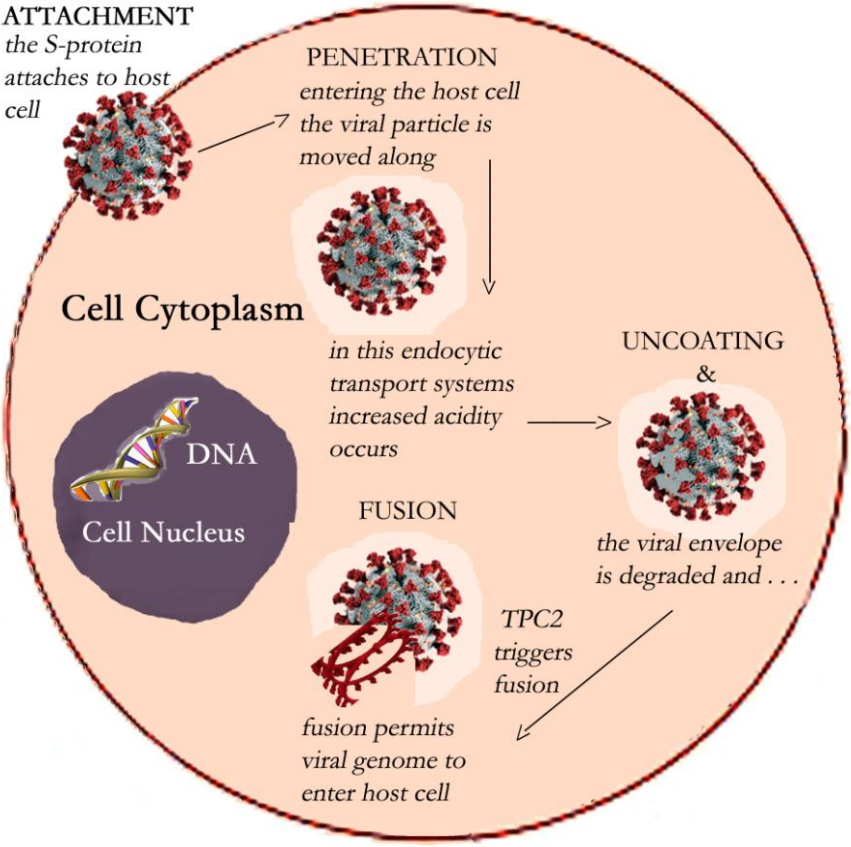
[213] Knyazev, Nersisyan, and Tonevitsky, "Endocytosis," 5; ACE2's cytoplasmic tail has an endocytic motif for a clathrin-adaptor subunit (AP2 $\mu 2$).
[214] Hu et al., "The Endosomal-Lysosomal System," 2.
[215] Jackson et al., "Mechanisms of SARS-CoV-2 Entry into Cells," 12, and 4.

3. Uncoating

Once the virus is inside the host cell, it undergoes a process of degradation called 'uncoating.' Transport of the penetrated virus is via packages in endosomes conducted along endocytic pathways. Along the way there is a gradual drop in pH (a measure of acidic/basic levels), in the direction of becoming more acidic. At last the bound viral particle reaches where it needs to be and a fusion pore can begin to open along the lines described in the previous chapter. But there we saw uncoating the virus inside the cell requires an intracellular receptor to bind and trigger this fusion. For SARS-CoV-2 a cellular factor named *lysosomal two-pore channel 2* (TPC2) typically induces fusion and subsequent uncoating,[216] or as we saw in endocytosis it may be cathepsin L-induced fusion.



After the viral nucleocapsid is released into the host's cytoplasm the viral genome serves as mRNA in the next phase (replication) of the viral cycle.[217]

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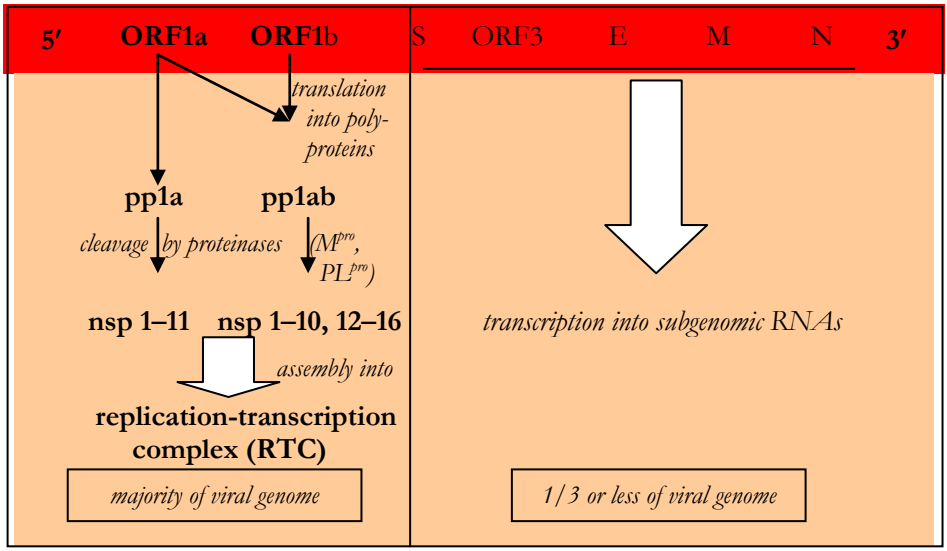
[216] Zhao, Qin, and Huang, "Lysosomal Ion Channels Involved in Cellular Entry," 1–2.
[217] De Breyne et al., "Translational Control of Coronaviruses," 12502.

4. Replication

Viral replication is intrinsic to infection. Peak infection by SARS-CoV-2 lasts about a week (5–7 days). During that time there will be 3–7 replication cycles, each of which involves 10–100 infectious units.[218] SARS-CoV-2 replication produces over time an abundant *viral load*—the measurable amount of virus in an infected person. It has been estimated that a person afflicted with COVID-19 carries between a *billion* (10^9) and a *hundred billion* (10^{11}) virions during peak infection. Adult men show a larger number than do adult women, and they in turn show more than do children. This volume still represents a small amount compared to the number of cells in a human body—less than 1%. Moreover, viral loads drop rapidly after the period of peak infection.[219]

The process of replication was broadly described in the previous chapter. We won't repeat that description except to begin with the illustration that summarizes it there, adjusted for some additional details.



As we've seen, the virus has attached and entered the host cell. Within the host cell's transportation system it experiences a form of degradation termed 'uncoating,' which releases the viral genome, or *genomic RNA* (gRNA). SARS-CoV-2 gRNA recruits host cell ribosomes and, now acting as mRNA, instructs those ribosomes as translation occurs of the principal Open Reading Frames (ORFs)—ORF1a and OR1b. These are translated into polyproteins.[220] But the translation is such that while ORF1a yields pp1a, it also contributes to ORF1b's production of a polyprotein, which is thus known as pp1ab.

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[218] Sender et al., "The Total Number and Mass of SARS-CoV-2 Virions," 6.
[219] Sender et al., "The Total Number and Mass of SARS-CoV-2 Virions."
[220] Malone et al., "Structures and Functions of Coronavirus Replication," 23.

The polyproteins pp1a and pp1ab subsequently undergo cleavage by proteases—a process known as *proteolysis*. For SARS-CoV-2 the main proteinase (M^{pro}) is a cysteine protease known as $3CL^{pro}$ (*3Chymotrypsin-like protease*, formerly known as C30 Endopeptidase). It cleaves pp1ab at 11 sites. The accessory proteinase PL^{pro} ('papain-like cysteine proteinase') cleaves it 3 sites.[221] Cleavage produces 16 nonstructural proteins (NSPs), with pp1a yielding nsp1 through nsp11, and pp1ab yielding nsp12 through nsp16.[222]

As seen briefly at the beginning of this chapter, the various NSPs play different functional roles. Nonstructural protein 1 (nsp1) is rapidly released and targets the host cell's translation machinery. The other fifteen (nsp2–16) compose the replication-transcription complex (RTC). With SARS-CoV-2, the RTC built features a few NSPs in especially notable roles. First in billing is nsp12, which functions as RNA-dependent RNA polymerase (RdRP), assisted by nsp 7 and nsp 8 as auxiliary factors.[223] Replication and translation also relies on nsp12, nsp13, nsp14, and nsp16.[224]

As just noted, the NSPs form the RTC and rearrange membranes of the endoplasmic reticulum (ER)—part of the intracellular transport network and involved in protein production. This rearrangement results in vesicles surrounded by a double membrane; it is within these packages that replication and transcription of SARS-CoV-2 occurs.[225]

The viral gRNA is a template RTC used to produce new gRNA and a set of subgenomic mRNAs (sg-mRNAs). The new gRNA—a full-length genome complement (the *anti-genome*)—itself serves as a template for replicating more gRNA. Through translation, it can yield more NSPs, serve as a template for further RNA synthesis, or be packaged in the assembly of new virions (see the next section). The sg-mRNAs are crucial because, through ORFs 2–9b, they provide templates for producing structural proteins (S, E, M, and N) and accessory proteins.[226]

SARS-CoV-2 replication not only has to ensure RNA synthesis, but also undertake proofreading to ensure the integrity of the genome, and modification/capping, a process that contributes to stability (modification) and adds a structure (capping) that prevents degradation while aiding evasion of host cell defense mechanisms. The NSPs for these actions are 7–8, 10, 12–14, and 16.[227]

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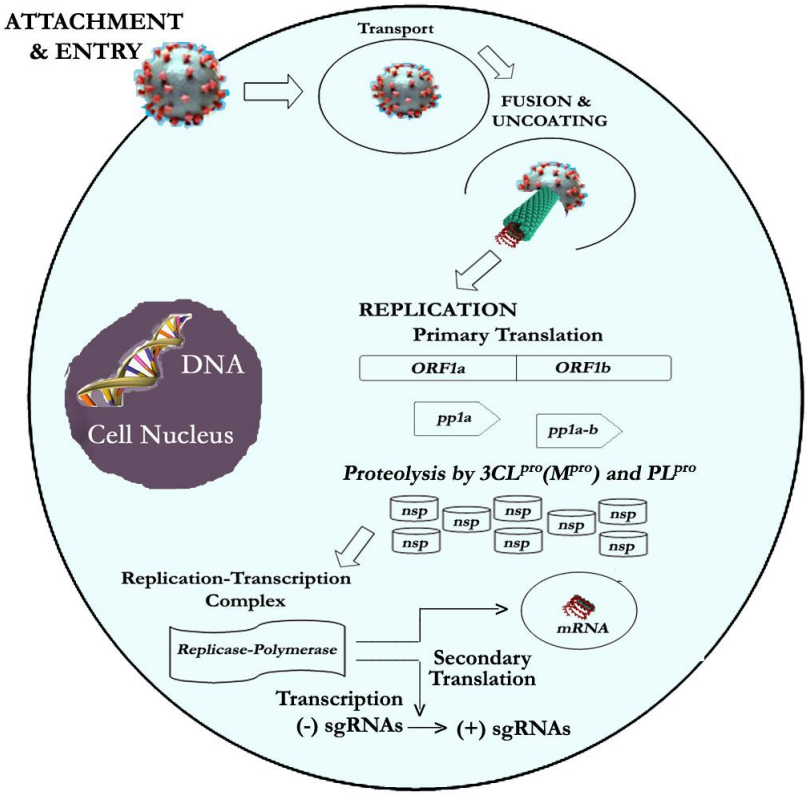
[221] Senger et al., "COVID-19: Molecular Targets," 5.
[222] Malone et al., "Structures and Functions of Coronavirus Replication," 24.
[223] Yan et al., "Architecture of a SARS-CoV-2 Mini Replication," 2.
[224] Malone et al., "Structures and Functions of Coronavirus Replication," 24.
[225] Pluskota-Karwatka, Hoffmann, and Barciszewski, "Reducing SARS-CoV-2 Pathological Protein Activity," 385. More specifically, as V'koski et al., "Coronavirus Biology and Replication," 156 [Fig. 1], note, the viral replication organelles of this micro-environment for replication consist of double-membrane vessicles (DMVs), convoluted membranes (CMs), and open double-membrane spherules (DMSs)—the last being small structures in the shape of a sphere.
[226] Malone et al., "Structures and Functions of Coronavirus Replication," 23 [Fig. 1], 24. Also see Li et al., "Human Coronaviruses: General Features," 4.
[227] Chen et al., "Synergistic Inhibition of SARS-CoV-2 Replication," 899.

The picture specific to SARS-CoV-2 looks like that shown in an illustration in the previous chapter, but here some additional detail is offered:

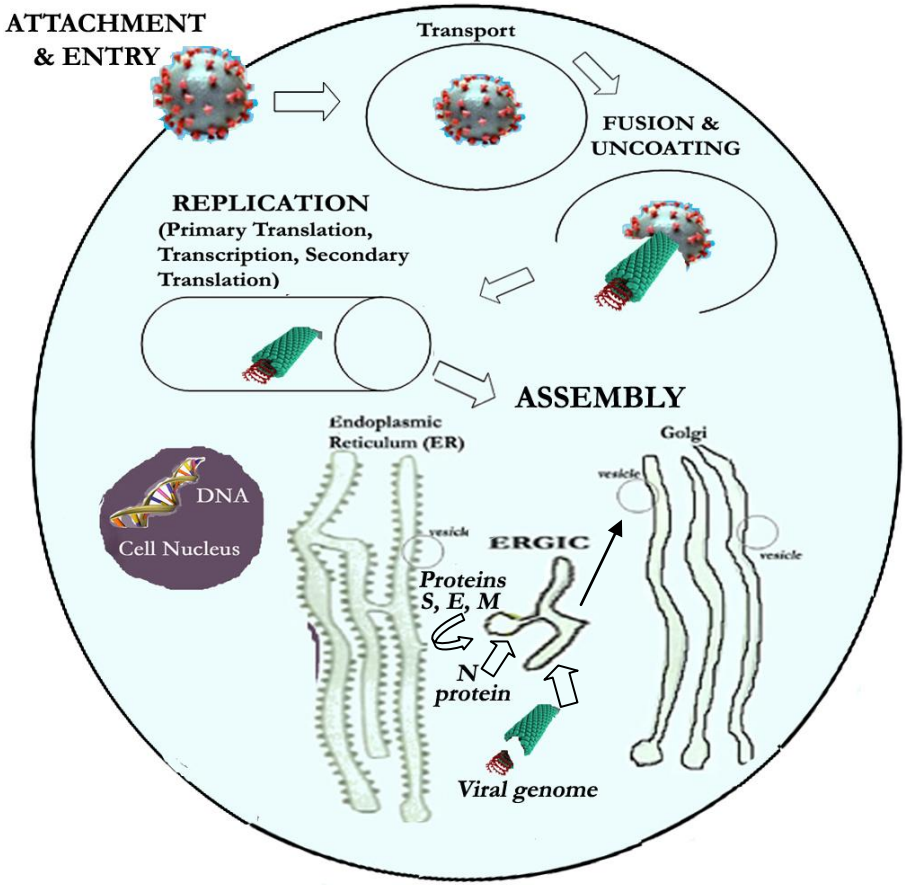


This replication process transpires in the host cell's cytoplasm, a realm filled with potential danger for SARS-CoV-2. The virus is exposed, at least potentially, to host cell defense mechanisms. Put metaphorically, though successful in breaching into the house through a door or window, there may be guards prowling inside looking for intruders. As mentioned incidentally above, SARS-CoV-2 meets this possibility in a variety of ways. One important way in replication is by co-opting host factors to form double membrane vesicles (DMVs). This rearrangement of the host cell membranes serves not only the structural scaffolding for viral RTCs, but also as protection against anti-viral host defenses.[228] In addition, a number of viral proteins inhibit the production by the host of interferons (proteins that inhibit viral replication) and cytokines (a category label for proteins that signal viral attack and signal the immune system).[229]

[228] Wong et al., "Exploiting Connections for Viral Replication," 1.
[229] Wong et al., "Exploiting Connections for Viral Replication," 6.

5. Assembly

New SARS-CoV-2 virions are assembled after replication. Let's start by returning to an earlier illustration (last chapter), once again expanded.



As shown above, the structural proteins S, E, and M at the ER membrane are joined to the structural protein N and the viral genomic material at ERGIC.

The process of assembly begins with reestablishing a protective coating of the new gRNA with the nucleocapsid (N) protein produced during replication. The nucleocapsid (N) protein is wrapped by the membrane (M) protein, with some involvement by the envelope (E) protein.[230] The new helical nucleocapsid structure then buds into the endoplasmic reticulum-Golgi intermediate compartment (ERGIC), in this process acquiring the other structural proteins (S, E, and M).[231] Thus the component parts are assembled and prepared for release.

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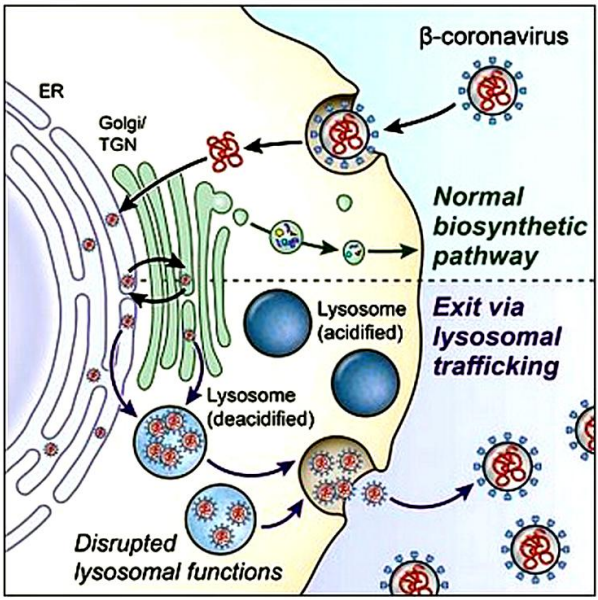
[230] Garoff, Hewson, and Opstelten, "Virus Maturation by Budding," 1171.

[231] Malone et al., "Structures and Functions of Coronavirus Replication," 23 [Fig. 1]. For a very brief summary of assembly, see Kirtipal, Bharadwaj, and Kang, "From SARS to SARS-CoV-2," 7.

6. Release

As noted in the last chapter, some coronaviruses (the β, or *betacoronavirus* family members) use lysosomes for exocytosis. SARS-CoV-2 is one such, though it presumably follows normal pathways, too. The illustration below contrasts the normal way—even for coronaviruses—that viruses are released from infected host cells with how some utilize a different path.[232]



The components of the normal biosynthetic secretory pathway (top) and the lysosome pathway (bottom).

(Image courtesy of National Institutes of Health (NIH))

When viral material needs release *cytopathic effects* (CPE) can be observed. Remember, these are changes wrought in the host cell by the virus to facilitate its release to infect other cells. In the cells lining the respiratory tract (the human airway epithelium) CPE is induced by SARS-CoV-2. So, too, is *apoptosis* ('programmed' cell death). CPE entail *budding* by which newly formed virus particles are provided nucleocapsids. The covered viral material is then placed in a package (*vesicle*) and released from the host cell cytoplasm through fusion of the vesicle with the cell body membrane. In this manner large quantities of SARS-CoV-2 are expelled to infect other cells.[233]

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[232] Ghosh et al., "β-Coronaviruses Use Lysosomes for Egress."
[233] See Zhu et al., "Morphogenesis and Cytopathic Effect of SARS-CoV-2."

SARS-CoV-2 Variants

Coronaviruses are liable to mutation. The original strain of SARS-CoV-2 was variously known—as Wuhan-Hu 1, as 2019-nCoV, and officially as SARS-CoV-2. Through mutation *variants*—groups sharing the same distinctive mutations—arise. Below is a list of some of the identified SARS-CoV-2 variants.[234]

WHO name	**PANGO designation**	**Identified as *Variant of Concern* (VOC)**	**Identified as *Variant of Interest* (VOI)**	**Identified as *Variant being Monitored* (VBM)**
Alpha	B.1.1.7 (and Q lineages)	12/29/2020		9/21/2021
N.10	B.1.1.33.10		4/6/2021 (Brazil)	
-	B.1.1.316			
-	B.1.1.318			
-	B.1.243.1			
Beta	B.1.351 (and descendants)	12/29/2020		9/21/2021
-	B.1.429.1			
Gamma	P.1 (and descendants)	12/29/2020		9/21/2021
P.2 *(see Zeta)*	P.2			
P.3	P.3			
Delta	B.1.617.2 (and AY lineages)	5/16/2021		
Epsilon	B.1.427 and B.1.429	3/19/2021	2/26/2021 6/29/2021	9/21/2021
Eta	B.1.525		2/26/2021	9/21/2021
Theta	B.1.1.28.3			
Iota	B.1.526 B.1.526.2		2/26/2021	9/21/2021
Zeta	P.2		2/26/2021	9/21/2021
Kappa	B.1.617.1		5/7/2021	9/21/2021
	B.1.617.3		5/7/2021	
Lambda	B.1.1.1.37			
Mu	B.1.621 and B.1.621.1			9/21/2021
Flurona	B.1.640.2			

[234] The material here draws principally upon the CDC (https://www.cdc.gov/coronavirus/2019-ncov/variants/variant-info.html#anchor_1632150752495) and the PANGO lineages (https://cov-lineages.org). Also see the WHO website (https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/)

As can be seen, most of the variant names are simply Greek letters in alphabetical order. The acronym PANGO stands for 'Phylogenetic Assignment of Named Global Outbreak Lineages'—created for epidemiological tracking of the lineages of SARS-CoV-2. The status of these lineages at any given time can be reviewed at cov-lineages.org. The variants are sometimes identified by four levels of classification, in ascending order of gravity:

- *variant being monitored* (VBM);
- *variant of interest* (VOI);
- *variant of concern* (VOC); and one not shown above—
- *variant of high consequence* (VOHC).

The classifications are fluid (as shown, for example, by the Alpha variant being a VOC in late 2020 but only being monitored as of Fall, 2021), and the designations shown above were current at the time of writing. These are for the United States unless otherwise indicated.

Variants, as noted above, share mutations. It has been estimated that, on average, a mutation is inserted into the SARS-CoV-2 genome every second infection.[235] Most variants display 20–30 mutations, with some notable examples (e.g., B.1.1.7 has nearly 40).[236] Although mutations provoke a measure of concern, coronavirus rates of mutation should be seen against those of other viruses. For example, coronavirus mutation rates are about half that of influenza. A typical coronavirus shows about two genetic letter changes a month in its genome (and recall that SARS-CoV-2's genome is about 29,900 nucleotides). Coronaviruses are, in the world of viruses, relatively stable. Even so, by the autumn of 2020 more than 12,000 mutations in SARS-CoV-2 had been documented. That number can seem alarming, but many mutations have no effect on such worrisome things as infectiousness or seriousness of symptoms. In fact, when mutations alter a viral protein they are more likely to harm the virus than the host.[237]

Decoding scientific notation on mutations can be challenging—and generally isn't of importance to nonspecialists. But it is worth a little comment. First, *the mutation under consideration might be in the genome in the RNA code*. In this respect, it has been reported that two mutation rates are significantly higher than others. These are G for U (guanine for uracil), and C for U (cytosine for uracil).[238] The significance of mutations in the genome depends on the impact they may have on an amino acid sequence in a protein.

Second, *the mutation might be in a protein, most especially the spike protein*. Let us consider an example. One mutation is known as 'Spike D614G.' It signals that the amino acid aspartate (D) was replaced by glycine (G) at the 614th amino acid

[235] Dejnirattisai et al., "Omicron-B.1.1.529," 6.
[236] Sender et al., "The Total Number and Mass of SARS-CoV-2 Virions," 7.
[237] Callaway, "Making Sense of Coronavirus Mutations," 175.
[238] De Maio et al., "Mutation Rates and Selection on Synonymous Mutations in SARS-CoV-2."

position of the spike protein. Thus letters stand for amino acids and the number for the position in the amino acid chain.[239]

Spike protein mutations appear far more common than mutations in any of the other viral proteins of SARS-CoV-2. For example, the Omicron variant that emerged in late 2021 shows 30 substitutions in the S protein—plus the deletion of 6 and insertion of 3 residues. Yet in all the other proteins there are a grand total of just 16 substitutions and 7 residue deletions.[240] The significance of the large number of mutations of S protein rests in that this is the viral structure used to attach to human host cells. Therefore preventive measures like vaccines naturally target this protein. But *the shifting nature of the S protein because of mutations constantly threatens vaccine efficacy*.

The PANGO lineage website provides far more information and has the advantage of being updated regularly. However, here we may note in passing certain variants showing mutations.

- The Alpha variant has been associated with the N501Y mutation.
- The Gamma variant has shown a number of spike mutations that are considered 'with likely functional significance' (E484K, K417T, and N501Y).
- The Theta variant has shown a number of spike mutations (E484K, N501Y, P681H, 141-143del.).
- The Iota variant (B.1.526), most predominantly detected in New York, is characterized by certain spike mutations (e.g., T95I and D253G), with spike mutation E484K present in about half its lineage (as of Feb., 2021).
- The B.1.1.316 variant shows spike mutation Q677H, and is parent to lineage R.1 and R.2 (which show mutations, including E484K).
- The B.1.1.318 variant shows spike mutations (Spike D614G, Spike D796H, Spike E484K, Spike P681H, Spike T95I, Spike Y144del).
- The B.1.243.1 variant emerged in Arizona and displays amino acid mutations (e.g., S:E484K and S:V213G).
- The B.1.429.1 variant shows Spike L453R mutation; predominantly circulating in California, it has also been detected abroad.
- The Delta variant AY.4.2 is characterized by spike mutations (Spike Y145H and Spike A222V).
- Omicron (B.1.1.529)—a highly mutated variant—was identified in late 2021. By early 2022, three subtypes had been identified.

The N501Y mutation seen in certain variants (e.g., Alpha, Gamma) is significant because it strengthens viral binding to the hACE2 receptor of host cells,[241] thus enhancing viral infectiousness.[242] The B.1.1.318 variant has aroused

[239] On this particular mutation, see Callaway, "Making Sense of Coronavirus Mutations," 174–75.
[240] Dejnirattisai et al., "Omicron-B.1.1.529," 4.
[241] Tian et al., "N501Y Mutation."

interest because of the numerous spike mutations and because it has been found in a widespread group of countries, including the United States, the United Kingdom, France, Germany, and Nigeria.

Delta Variant

The Delta variant was first identified in India in late 2020 and quickly became the dominant strain in the world. Entering December, 2021, it was estimated to account for 99.9% of all SARS-CoV-2 detected in the United States.[243]

Because of its dominance, the Delta variant continued to receive significant attention until late in 2021 when it began to be displaced. The Delta variant AY.4.2, though still in the autumn of 2021 less common than variant AY.4.4, was increasing in number of infections in the United Kingdom. But it was also showing signs of producing fewer symptoms while being no more resistant to vaccine than the AY.4.4 variant. It has been suggested that, if true, the increase in infection rate by this variant may be due to more asymptomatic carriers spreading the virus.[244]

Omicron Variant

On the other hand, just as there were signs that Delta might be mutating to a less dangerous form, around Thanksgiving, 2021, a new variant—B.1.1.529—spread alarm through much of the world. This variant is highly mutated compared to earlier variants. Omicron showed more than 30 mutations of the spike protein—twice as many as Delta and more than any other variant to date. Of the 37 mutations identified, 26 (23 substitutions, 2 deletions, 1 insertion) are distinct when compared to other variants.[245] A mid-December, 2021, preprint reported four spike mutations (S371L, N440K, G446S, and Q493R) that appear to confer greater antibody resistance.[246]

Analysis of Omicron's genome has raised the possibility that its greater infectiousness, with greater resistance to immune system detection, may result from some template switching with the genomes of other viruses that infect the same cells, or the human transcriptome of host cells infected with SARS-CoV-2.[247] By the end of December analysis of Omicron was leading to speculation that the variant may have evolved to escape neutralization by Beta variant specific serum (Beta had been displaced in Southern Africa by Delta). Specifically, research was indicating that changes at the RNA-binding domain (RBD) of the spike (S) protein of SARS-CoV-2 were enabling strong enough attachment to human ACE2 receptors on host cells to provide significant immune escape.[248]

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[242] Liu et al., "N501Y Mutation."
[243] CDC, "SARS-CoV-2 B.1.1.529 (Omicron) Variant," 1731.
[244] Browne, "What We Know about Delta AY.4.2 COVID Variant."
[245] Venkatakrishnan et al., "Omicron Variant," 2.
[246] Liu et al., "Striking Antibody Evasion Manifested by the Omicron Variant, 7."
[247] Venkatakrishnan et al., "Omicron Variant," 3.
[248] Dejnirattisai et al., "Omicron-B.1.1.529," 3–6, and especially pp. 25–26. Also see Reinicke et al., "SARS-CoV-2 Beta Variant."

What may have fueled the emergence of Omicron was *vaccine inequity* (disparities in who receives the opportunity of vaccination) and *vaccine hesitancy*.[249]

Another hypothesis floated at the end of the year was that Omicron may have developed inside immune-compromised people infected by SARS-CoV-2. For example, in HIV-positive individuals who are not taking their medications for the disease, infections of COVID-19 sometimes linger for months—a prolonged period during which the virus can undergo significant mutations. The thinking is that these immune-suppressed hosts facilitate prolonged infection, giving rise to more replication cycles, leading to more mutations. In this respect, certain case studies have drawn special attention. For instance, in one a South African woman is known to have tested positive for COVID-19 for almost eight months, and the lead researcher of the team who identified the Omicron variant notes some 10–15 similar cases had been found in other places around the world. If diseases that suppress the immune system provide hosts for SARS-CoV-2 where lingering infection provides opportunities for mutation, then it becomes all the more important to shield such people from infection and to encourage in them compliance with the medications prescribed for them.[250]

Infectiousness & Spread

Research reported in mid-December, 2021, found that at 24 hours after infection, Omicron multiplies *70 times faster* than Delta or Wild Type (i.e., the original strain) in the human bronchi (the tubes conveying air from the windpipe to the lungs).[251] This infectious capability led to extraordinarily rapid spread.

Believed to have originated in Botswana, in Southern Africa, it quickly spread to South Africa, Hong Kong, Israel, and Belgium. First alerted to this variant on November 24, 2021, on November 26th WHO assigned the new variant the name 'Omicron' and identified it as 'a variant of concern' (VOC).[252] This was followed by the U.S. SARS-CoV-2 Interagency Group (SIG) also declaring it a variant of concern on November 30—one day before the first recorded case in the U.S. was reported.[253] Within days it had been detected in a dozen nations and by December 1, 2021, in some 20 nations, including Canada and the United States. By mid-December the variant had been found in about half of the states of the U.S.;[254] by late December in all but four of them. Similarly, at that time some 89 nations reported finding the variant within their borders. By late December it was the dominant variant in the United States and many other places.

An early study in South Africa attested the infectiousness of Omicron—it rose in just two months from 3% of cases in early October to 98% of cases in

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[249] Venkatakrishnan et al., "Omicron Variant," 4.
[250] Harding, "Omicron: South African Scientists Probe Link."
[251] LKS Faculty of Medicine, "HKU Med Finds Omicron SARS-CoV-2 Can Infect Faster."
[252] Callaway, "Heavily Mutated Coronavirus Variant," and Connor, "Scientists Raise Alarm."
[253] CDC, "SARS-CoV-2 B.1.1.529 (Omicron) Variant," 1731.
[254] In 22 states by Dec. 8th. CDC, "SARS-CoV-2 B.1.1.529 (Omicron) Variant," 1731.

early December.[255] By about the same time it was the dominant variant in England, where the concentration of cases was among young adults (ages 20–29).[256] In the United States, the dramatic rise in Omicron infections prompted the Institute for Health Metrics and Evaluation (IHME) at the University of Washington to revise its projections for the spread of COVID-19. They estimated infections would rise from below 19 million on December 22, 2021, to more than 38 million by January 31, 2022—a doubling in a span of just five weeks.[257] The issue of reinfections was also emerging as a significant concern; in England 9.5% of Omicron cases were reinfections, across all ages—and this was considered a serious underestimate.[258]

Severity

Though more infectious than Delta, early reports suggested it might be associated with generally milder symptoms. Patients with COVID-19 caused by the Omicron variant typically experienced fatigue, head and body aches, occasional sore throats, and coughing.[259] The South Africa study cited above reported that the 29,721 infections with Omicron resulted in a smaller percentage of hospitalizations compared to Delta. Omicron infections were associated with 80% lower odds of hospitalization and 70% lower odds of severe disease.[260] The United Kingdom (UK) Health Security Agency also reported in late December an apparently reduced risk of hospitalization compared to Delta (60% less). However, hospitalizations and deaths do occur.[261]

Affirming hopes that Omicron may be less severe, the same study reported above concerning infection rate versus Delta also found Omicron infection in the lungs is at least *10 times less* than Wild Type. Nevertheless, as lead researcher Michael Chan Chi-wai warns, a very infectious variant even though less pathogenic may still cause more instances of severe disease and death than a less infectious strain simply because there are so many more people infected.[262]

In the United States, early data from 37 symptomatic patients infected by the Omicron variant was summed up by the CDC this way: "Many of the first reported cases of Omicron variant infection appear to be mild, although as with all variants, a lag exists between infection and more severe outcomes, and symptoms would be expected to be milder in vaccinated persons and those with previous SARS-CoV-2 infection than in unvaccinated persons."[263]

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[255] Wolter et al., "Early Assessment of the Clinical Severity,"
[256] UK Health Security Agency, "SARS-CoV-2 Variants" (Technical Briefing 33), 3.
[257] IHME projections (Dec. 22, 2021) at https://covid19.healthdata.org/global?view= infections-testing&tab=trend&test=infections.
[258] UK Health Security Agency, "SARS-CoV-2 Variants" (Technical Briefing 33), 4, 28.
[259] Sguazzin, "Doctor Who Saw Omicron Early Says Symptoms Different to Delta."
[260] Wolter et al., "Early Assessment of the Clinical Severity," 9–10.
[261] UK Health Security Agency, "SARS-CoV-2 Variants" (Technical Briefing 33), 4, 21, 23; 14 people had died, with a median time from specimen date to death of 4 days (range 1–10 days).
[262] LKS Faculty of Medicine, "HKU Med Finds Omicron SARS-CoV-2 Can Infect Faster."
[263] CDC, "SARS-CoV-2 B.1.1.529 (Omicron) Variant," 1733 (see Table 3 for data used here). .

The extremely rapid spread of the new variant, coupled with so many continuing to resist vaccination, led to renewed concerns about the impact of Omicron. This was especially true in the United States where vaccine resistance continued to result in a heavy toll. In late December, 2021, IHME projections were that hospital usage in the U.S. would more than double by mid-February and daily death totals might do likewise.[264] In early 2022 (January 11) IHME was projecting 905,265 reported COVID-19 deaths by May 1, 2022.[265]

Vaccine Efficacy against Omicron

Rapidly spreading new variants inevitably raise concerns about vaccine effectiveness.[266] Although highly preliminary in late 2021, initial evidence suggested worrisome trends. Omicron was showing itself so infectious that neither vaccination nor previous infection seemed to slow it down. The CDC reported that of 43 cases of Omicron infection it had examined, 34 (79%) had occurred in persons vaccinated at least two weeks before symptom onset or receipt of a positive SARS-CoV-2 test result—and of those 34, 14 (41%) had received a booster dose, though 5 of them had received the additional dose less than 14 days before symptom onset. In addition, 6 (14%) persons had a documented previous SARS-CoV-2 infection.[267] Taken together these preliminary numbers suggested Omicron was so different in nature that no measure of previously conferred immunity, whether by infection or vaccination, was much defense.

The UK Health Security Agency found after repeated analysis of vaccine effectiveness that it was lower for symptomatic Omicron compared to Delta. Even with a booster shot there appeared to be a 15–25% reduction in effectiveness after 10 weeks.[268] In Sweden, in research conducted at the Karolinska Institute, a study involving 17 previously infected health care workers and 17 Swedish blood donors found reduced levels of Omicron-neutralizing antibodies.[269]

A preprint paper noted neutralization titres in people who had received the AstraZeneca or Pfizer vaccine showed significantly lower levels against omicron than against earlier variants Beta and Delta.[270] Similarly, early reports on the widely used Chinese vaccine *CoronaVac* found that even a booster shot after two doses did not produce a sufficiently robust response to Omicron.[271] This lessened effectiveness was attributed to the mutations characterizing Omicron.

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[264] IHME projections (Dec. 22, 2021) at https://covid19.healthdata.org/global?view= infections-testing&tab=trend&test=infections. The late December projection was from about 6900 to about 7400, compared to a range of from about 6,000 daily deaths (*if* there were 80% mask use) to slightly more than 1400 daily deaths with a high severity of Omicron.

[265] IHME projections (Jan. 11, 2022) at https://covid19.healthdata.org/united-states-of-america?view=cumulative-deaths&tab=trend.

[266] For a mainstream media summary, see Dunn and Schuster-Bruce, "Here's the Data."

[267] CDC, "SARS-CoV-2 B.1.1.529 (Omicron) Variant," 1731–32.

[268] UK Health Security Agency, "SARS-CoV-2 Variants" (Technical Briefing 33), 4, 24–27.

[269] Calloway, "Omicron," 368.

[270] Dejnirattisai et al., "Reduced Neutralization of SARS-CoV-2."

[271] Hong, "Three Sinovac Doses Fail to Protect."

Another study, reported in mid-December by South Africa's largest private health insurance administrator—Discovery Health—examined 211,000 cases of COVID-19. This preliminary study found that the effectiveness of Pfizer's vaccine against infection was significantly reduced against Omicron (from 80% against earlier variants to 33% against Omicron). However, full vaccination still provided significant protection (70%) serious illness resulting in hospitalization (compared to 93% against earlier variants).[272]

Another preprint paper in mid-December found Omicron "markedly resistant" to neutralization and that this was true even in those fully vaccinated. They found that the activity of 18 of the 19 antibodies they tested was either abolished or impaired. They concluded the variant poses "a serious threat" to existing vaccines and treatments.[273]

On the other hand, a preprint study reported in late December that booster shots of AstraZeneca's vaccine or the Pfizer mRNA vaccine significantly boosted antibody levels against Omicron. Antibody levels after the booster were higher than those found in individuals with 'natural immunity' from infection by the original strain or earlier variants (Alpha, Beta, or Delta). This research suggested that while development of a vaccine specific to Omicron might occur, current vaccines, especially after a booster dose, still show high antibody levels and indicate substantial protection against severe illness.[274]

New Variants and the Future

Yet another variant was identified in late December, 2021, in a person arriving in France from Cameroon. This variant has even more mutations than Omicron—46, including S protein mutations N501Y (which facilitates binding to host cells) and E4484K (which facilitates escape from immune system detection).[275] In January, 2022, researchers on Cyprus reported a variant one dubbed 'deltacron' because of its Omicron-like genetic signature within Delta genomes.[276] *RNA viruses mutate; expect more variants to emerge.*

However, viruses often become less deadly over time—even if they become more infectious. It is not in the virus' best interests to kill too many hosts. A study on this matter, covering four waves of the pandemic, found increases in daily infections but not in the magnitude of mortality. In other words, infections increased more than did deaths. Factors affecting mortality rate include virus mutations and vaccinations. Both seem to contribute to infections staying high but the risk of death often declining.[277]

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[272] Discovery Health, "Discovery Health."

[273] Liu et al., "Striking Antibody Evasion Manifested by the Omicron Variant."

[274] Dejnirattisai et al., "Omicron-B.1.1.529," 12–18, 28–29. Cf. AstraZeneca, "Vaxzevria Significantly Boosted Antibody Levels against Omicron."

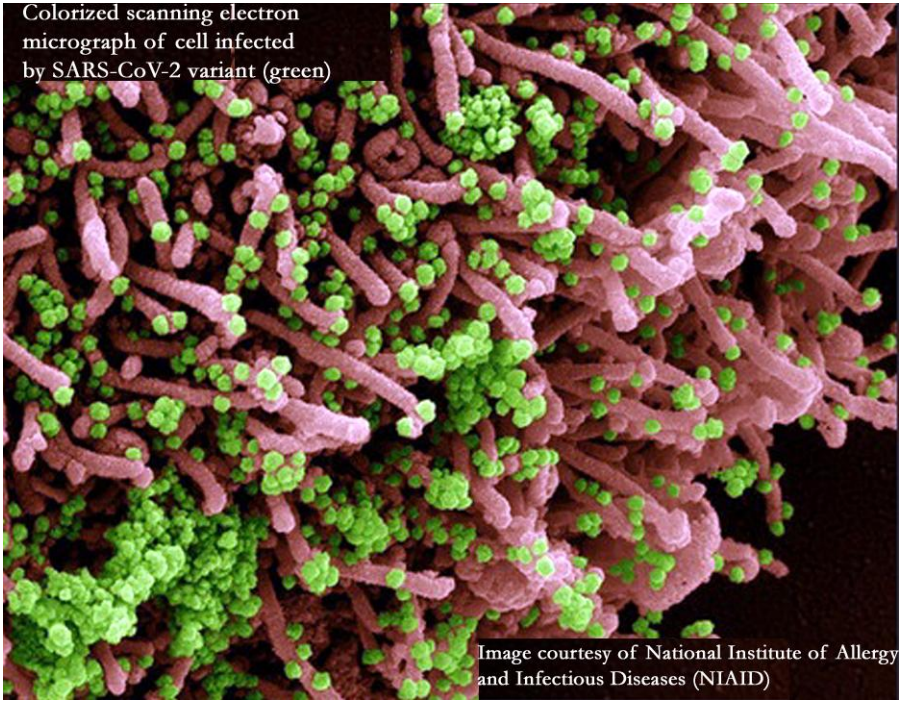
[275] Freund, "New Corona Variant."

[276] Bursztynsky, "Cyprus Reportedly Discovers a COVID Variant that Combines Omicron and Delta."

[277] Zabwaa et al., "Effect of Mutation and Vaccination." Mortality rates vary among nations.

Chapter 4
COVID-19

The SARS-CoV-2 virus is termed *pathogenic* because it is the origin (*-genic*) of suffering (*path-*). It is the cause of the disease named COVID-19, to which we now turn. The name 'COVID-19' is simple enough: 'Co' is for 'corona,' 'vi' is for 'virus,' and 'd' is for 'disease,' with '19' short for '2019.' Thus, COVID-19 means the coronavirus disease identified in 2019. It was referred to at first as a 'novel' coronavirus, because it was *new*, having not been previously known. Although it was circulating before it became publicly identified, once it was named as SARS-CoV-2 (February 11, 2020) the disease whose spread it prompted was quickly named COVID-19 by the World Health Organization (WHO).



Brief History of COVID-19

SARS-CoV-2 causes the disease named COVID-19, first noticed in December, 2019. The disease spread from Wuhan, the capital of Hubei province in central China (which is why it also has been known as Wuhan-Hu 1). The onset of the first recorded case there was December 8, 2019. However, attention began to occur with the report at the end of that month of more than two dozen pneumonia-like cases of illness in Wuhan. Less than two weeks later (January 9,

2020) the Chinese government reported the cause of these cases to be a previously unknown—'novel'—coronavirus.[278]

The new 'Wild type' coronavirus proved to be extraordinarily infectious. Within a month it had been found in all 34 provinces of China. By the end of January the World Health Organization (WHO) declared it a public health emergency of international concern. By then the disease had escaped Chinese boundaries. It was detected in Thailand in mid-January, having been conveyed there by a traveler from Wuhan. Even as the situation worsened in China, where by mid-February there were some 3,000 new cases a day, international travel facilitated the global spread of the disease. On March 11, 2020, just three months after the first putative case, WHO declared the worldwide outbreak a *pandemic*—literally, an epidemic affecting all (*pan-*) the places people live.[279]

The pandemic showed fluctuations of enough significance they came to be referred to as 'waves.' These surges in infection numbers accompanied the ascendance of new virus variants (Alpha, Beta, Delta, Omicron). Pandemics are easily associated with acute respiratory tract infections (ARTIs) like COVID-19 because such infections are the most common—and already this century no less than three serious coronavirus diseases of this nature (SARS, MERS, and COVID-19) have appeared. But while often outbreaks are localized—epidemics—when new ('novel') viruses emerge and are highly infectious they spread rapidly before adequate immunity in the population can be accrued.[280]

The virus was quickly and extensively studied, with the first genome sequence published January 10, 2020, and more complete genome sequences two days later. But there was a sense of scrambling to catch up to a deadly disease that continued to spread rapidly. By mid-August of 2020, COVID-19 had been found on six continents in 216 countries. More than 20 million cases had been reported in just nine months of the disease, with more than 733,000 dead.[281] By the time the Christmas season of 2021 had arrived nearly 278 million cases had been reported worldwide, with nearly 5.4 million dead.[282] In the United States—a world leader in cases and deaths through much of the pandemic due to 'vaccine hesitancy' fueled by anti-vaccine fear mongers—there were more than 51 million cases and more than 800,000 dead.[283]

By any measure the cumulative effect of COVID-19 has been significant. At the end of 2021 it stood as the fourth deadliest pandemic in recorded history (after smallpox, the 1918/19 flu, HIV/AIDS, and plague).[284]

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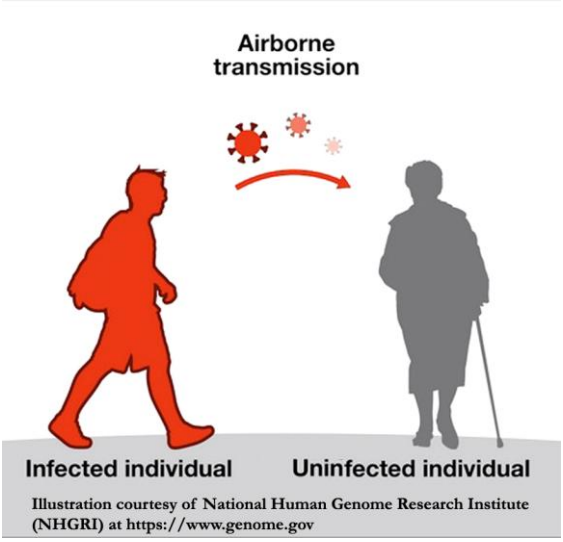
[278] Hu et al., "Characteristics of SARS-CoV-2 and COVID-19," 141–42.
[279] Hu et al., "Characteristics of SARS-CoV-2 and COVID-19," 142.
[280] Khan et al., "COVID-19: A Global Challenge," 2.
[281] Hu et al., "Characteristics of SARS-CoV-2 and COVID-19," 141–42.
[282] Worldometer (Dec. 23, 2021), at https://www.worldometers.info/coronavirus/ reported 277,891,348 cases with 5,397,228 deaths.
[283] CDC, "COVID Tracker" (Dec. 23, 2021), at https://covid.cdc.gov/covid-data-tracker/ #datatracker-home. The tracker is updated daily. The exact numbers were 51,324,667 cases with 807,397 deaths. On the US rank, see Khan et al., "COVID-19: A Global Challenge," 15 (Table 1).
[284] Khan et al., "COVID-19: A Global Challenge," 6 (Fig. 2).

By now almost everyone has heard so much about COVID-19 that they are aware that SARS-CoV-2 virus is largely airborne between infected persons and uninfected folk; *human to human transmission* is the primary way the virus is spread (as shown in the illustration at right).



However, this idea of airborne transmission can be a little misleadingly if one infers from it that the primary cause of infection is from breathing in the virus—direct airborne inhalation of SARS-CoV-2.

As viral particles are loosed from an infected person they *can* be directly inhaled—but this seems not to be the most common avenue for infection. Airborne particles can be deposited in mouth, nose, or eyes by 'splashes or sprays' such as those that occur when someone coughs on another, and that is a much more likely infectious path. This is why masks, which hinder or stop splashes or sprays, are a sensible measure. Less likely, but possible, is contact with viral particles on surfaces that are then conveyed by the hands to the eyes, nose or mouth.[285]

By the end of 2021 there seemed to be an emerging consensus that five potential modes account for most if not all of SARS-CoV-2 transmission:

- airborne inhalation of virus in aerosols;
- droplets;
- contact with contaminated surfaces;
- oral secretions; and,
- fecal secretions.[286]

Of the five transmission modes just listed, droplets seems to be the most common avenue for infection, followed by inhalation of aerosols.[287] Rather than being discrete categories, aerosols and droplets are best conceived as situated along a continuum and both here are referring to products of respiration. It has become commonplace to distinguish between aerosols and droplets based on

[285] See CDC, "SARS-CoV-2 Is Transmitted by Exposure."
[286] Mehraeen et al., "Transmission Modes of COVID-19."
[287] Zhou et al., "Modes of Transmission of SARS-CoV-2," 1.

size, with 5 micrometers (μm) used as the dividing line.[288] What can confuse the picture even more for an unwary reader is that 'aerosol' is sometimes used in a broader manner such that 'droplets' becomes a subcategory.[289]

Respiratory aerosols are smaller particles (<5 μm) and can remain airborne indefinitely in indoor spaces where there is little circulation of air or ventilation. In such environments, virus-containing aerosol can persist for long periods and at high concentrations—factors further increasing the rate of transmission.[290] *Infectious aerosols* are well-described as "suspensions of pathogens in particles in the air, subject to both physical and biological laws." Aerosol plumes can be generated by coughing or sneezing and carry up to 26 feet (8 meters).[291] Research has determined that SARS-CoV-2 virions range in size from 0.07–0.09 μm, which means that if only 1% of a respiratory fluid particle is occupied by the virus, a particle the size of 0.4 μm can contain it. In the real world, there is variability in particle sizes, which influences how long they remain airborne, but the bottom line is that transmission through aerosols is quite possible.[292]

Respiratory droplets are larger particles (>5–10 μm). Because they are larger and weightier they drop out of the atmosphere more quickly and thus do not remain airborne as long as aerosols. However, a moment ago we saw that coughing and sneezing can produce plumes that travel quite a distance under favorable conditions. Here we must add that coughing and sneezing can generate clouds of largely liquid particles commonly ranging in size from 1–100 μm, meaning they might be in either 'aerosol' or 'droplet' form as we are distinguishing those two means of transmission. Droplets, not unexpectedly, show larger amounts of virus ('viral loads')—those from 10–100 μm on average have 1,000 times greater load than the smaller aerosol particles. And even at this larger size, given favorable enough conditions, these droplet particles can travel over 98 feet (30 meters).[293]

Contact with contaminated surfaces was a focus of much attention early in the pandemic. It remains a potential mode of transmission but is not today seen as worrisome as feared earlier. In optimal conditions (68°F/20°C, relative humidity of 50%) research suggests SARS-CoV-2 can persist about 28 days on some surfaces (e.g., paper money, glass), and up to 21 days in hospital settings on some personal protective equipment (PPEs) such as N95 mask material. But such things were measured in the laboratory. In the real world, the virus is sen-

[288] For the history of this development see Randall et al., "How Did We Get Here," esp. 5–6.
[289] Gorbunov, "Aerosol Particles," 1, refers to "an ambiguity about terminology" in which the terms 'droplet' and 'aerosol' may be used by different authors for the same kind of particle.
[290] Rahman et al., "The Transmission Modes and Sources of COVID-19," 128.
[291] Fennelly, "Particles Sizes of Infectious Particles," 914.
[292] Lee, "Minimum Sizes of Respiratory Particles," 3–6.
[293] Gorbunov, "Aerosol Particles," 4.

sitive to changes in either temperature or relative humidity with increases in either proving detrimental to the virus. Sunlight rapidly inactivates it.[294]

On the other hand, *oral and anal secretions*—particularly the latter—have increasingly gained attention. Whereas the virus is typically cleared from the respiratory tract within a couple of weeks, the feces can remain positive for viral RNA for longer than 4 weeks. This helps explain concern over sharing toilets.[295]

A moment ago temperature and humidity were referred to as elements that can affect transmission. That idea leads us to a broader consideration. *Weather* and infectious diseases are linked by airborne transmission. In a study of reports on this issue, researchers found that a complex relationship appears to exist between incidence of COVID-19 and weather variables. *Temperature* emerged as the most consistently reliable variable for predicting incidence of COVID-19, and *relative humidity* the second best predictor. In general, SARS-CoV-2, like other airborne viruses, does best when temperatures and relative humidity are both low.[296] One study found that as the air immediately surrounding people ('ambient' temperature) warms, the number of COVID-19 cases decreases (1.44 cases per day for each 1° Fahrenheit increase). On the other hand, as precipitation increases, so do COVID-19 cases (56.01 cases/day for each inch increase in precipitation).[297] Both of these correlations make sense with the idea we started with in this paragraph: nice weather draws people outside while inclement weather brings them inside where virus transmission is facilitated.

By far *the largest factor in virus transmission is human behavior*. Regardless of external factors such as weather, the primary conduit of transmission remains person-to-person contact, whether directly (as in conversation) or indirectly (as in virus left in the air or on surfaces by an infected person). Sunil Thomas, a researcher with a background in microbiology, immunology, molecular biology and cell biology, puts the matter simply and clearly: "The primary route of transmission of COVID-19 is likely via respiratory droplets and is known to be transmissible from pre-symptomatic and asymptomatic individuals. Infected people spread viral particles during talking, breathing, coughing, or sneezing." He points out that viral particles might be encapsulated in globs of varying kinds (mucus, saliva, water), and both how they behave and how long they persist in any given setting depends on the size of the globs. For example, he notes, studies show that SARS-CoV-2 can be detected and remain viable 3 hours after being let out into the air.[298]

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[294] Riddell et al., "The Effect of Temperature on Persistence," 3, 5. This research was conducted in laboratory settings and not in real world ones where actual viral viability seems lower.
[295] Rahman et al., "The Transmission Modes and Sources of COVID-19," 129.
[296] McClymont and Hu, "Weather Variability and COVID-19." Yang, Li, and Cao, "Influence of Meterological Factors," 7, note that "virus activity and enzymes necessary to replicate in the host are mainly influenced by the highest, lowest optimum temperatures, instead of day–night temperature difference."
[297] Sobral et al., "Association between Climate Variables and Global Transmission."
[298] Thomas, "The Structure of the Membrane Protein," 348.

Susceptibility of Infection

In the preceding chapter we examined the process of infection of human host cells by SARS-CoV-2. Here we can look at the disease side, that is, *the experience of infection* aside from the matter of symptoms (discussed later).

Likelihood of Infection in the United States

Before we can talk about the actual experience of infection we must address the *likelihood* of infection. Determining this has not been easy. Anti-vaxxers have consistently argued that the risk of infection has been exaggerated by the government, as well as misrepresented in official reports. Epidemiologists think otherwise, arguing that the numbers are under-reported.

A study in the journal *Nature* identified three 'waves' of the pandemic in the United States in 2020 (spring, summer, autumn/winter). Officially, by the end of 2020 more than 19.6 million cases of the disease had been reported. But the researchers in this study also determined that some 78.2% of all infections during the year went undocumented. Using a county-resolved metropolitan model for the 3,142 counties in the nation, they estimated that by the end of the year about one-third (31%) of Americans had been infected by COVID-19 (and that estimate was without considering reinfections).[299]

In the fall of 2021, Dr. Amesh Adalja, an infectious disease specialist at Johns Hopkins Center for Health Security, said in an interview, "It's likely that everybody will probably get infected with COVID-19 [at some point] because it's an endemic respiratory virus."[300] Of course, not every expert may agree on that point, but the general conclusion among them is that infection by SARS-CoV-2 is far more likely than anti-vaxxers want people to believe.

Influence of Coronavirus Variants

One significant factor in the likelihood of infection is which SARS-CoV-2 variants are dominant in the area in which a person resides. Some variants have proven more infectious than others. A journal pre-proof of an article late in 2021 for the journal *Cell* reported on four variants of concern and how they compared to the 'wild-type' (original SARS-CoV-2 virus identified). With respect to *transmissibility*—the likelihood of infecting others—they reported:

- Alpha variant is estimated to be *43–100% more transmissible.*
- Beta variant is estimated to be *up to 50% more transmissible.*
- Gamma variant is estimated to be *70-140% more transmissible.*

They also reported *Delta variant as at least 60% more transmissible than Alpha variant.*[301] Then the Omicron variant emerged—and it proved dramatically more infectious than Delta (as described in the previous chapter).

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[299] Sen et al., "Burden and Characteristics of COVID-19."
[300] Quoted in Cohen, "Breakthrough COVD-19 Cases."
[301] Bushman et al., "Population Impact of SARS-CoV-2 Variants," 1–2 {Journal pre-proof].

Individual Factors

Individual vulnerability is something early acknowledged in the pandemic. In this section our concern is principally with risk factors for severe disease, although some are factors related to risk of infection, too. A number of factors have been implicated by research. Here are fifteen:

- *age*—increased age indicates increased vulnerability;
- *behavioral*—behaviors like smoking increase risk;
- *blood type*—Type A seems more susceptible;
- *body mass index* (BMI)—those obese are more vulnerable;
- *environment*—proximity and person factors;
- *ethnicity and race*—minority groups are more vulnerable;
- *genetics*—specific locations on the human genome;
- *geography*—greater vulnerability associated with northern climes;
- *pregnancy*—being pregnant *might* increase risk;
- *psychological factors*—those prone to anxiety/fear more vulnerable;
- *sex*—males more vulnerable;
- *societal factors*—the poor are more vulnerable;
- *underlying medical conditions*—immunocompromised conditions, etc.;
- *vaccination status*—the unvaccinated are at more risk; and,
- *vitamin D*—vitamin D deficient people are more vulnerable.

Although the above factors indicate relatively greater vulnerability, none of them guarantee infection upon exposure, just as the absence of all these factors does not guarantee noninfection upon exposure.

Space limitations mean we cannot engage in any thorough analysis of these factors here. Instead, our examination is meant to briefly substantiate their importance as risk factors, explain why, and provide a taste of the scientific literature. Additional research is strongly encouraged, especially since as the pandemic continues more data is accrued and increased analysis yields further insights. In addition to the following remarks concerning these factors that increase risk of severe disease are some comments at the end of this part on possible risk modifiers.

Age Factors

All of us have heard regularly about the increased risk of severe COVID-19 in the elderly because of decreased immunological protection due to normal aging. Coupled with this is the factor of underlying medical conditions, which are more likely in the elderly. Nevertheless, as one systematic review and meta-analysis of the scientific literature reported, "It was extremely difficult to find age ranges to assess risk of mortality since there was wide variability in reporting clinical outcomes with age ranges." In fact, out of 20 studies only 6 reported data in such a manner that allowed the researchers to group patients into two broad divisions—those age 50 or older and those under age 50. Doing so re-

vealed a 15.4-fold increased relative risk of death in the older group—a disproportionate outcome in light of having only a 3.45-fold greater risk of infection by SARS-CoV-2.[302]

There seems little need to examine many studies on this widely reported observation of increased risk for the elderly. However, at least a quick peek at some empirical data is always warranted. A meta-analysis of 59 studies comprising 36,470 patients in 8 countries found that age 70 proved a useful marker for comparing relative risk of infection (1.65x greater risk—65% higher—over age 70), developing severe illness (2.05x greater), being admitted to an Intensive Care Unit (2.70x greater), and dying from COVID-19 (3.61x greater).[303]

But as the pandemic has persisted, more attention has been drawn to other age groups. In trying to understand how infection spreads, a study of 767 'hot spot' counties (24% of all U.S. counties, with 63% of the nation's population) during the period June 1–July 30, 2020, found *increases in documented cases of COVID-19 started in younger age groups*. The increases in cases within age groups then came in shorter and shorter times before hotspot status. This shows the trend:

Age Range	Days before 'Hotspot' Status that Increase in Cases Documented	Percent of Positive PCR Tests during 'Hotspot' Period
0–17	31	11%
18–24	31	14%
25–44	28	10%
45–64	23	8%
65+	20	6%[304]

(PCR tests are done to establish whether a SARS-CoV-2 infection is present, and quite often an infection is not, which yields a negative result.) Note that the 18–24 age group emerged earliest (along with the 0–17) and had the highest percentage of positive test results during the hotspot period.

A study published in *Science* found that by mid-2020 the resurgence seen in COVID-19 numbers was mostly driven by people ages 20–49 years old (and especially those 35–49). In fact, at the end of October, 2020, the two age groups 20–34 and 35–49 were the only ones in the U.S. sustaining COVID-19 with transmission rates consistently above 1.0 (a rate of 1.0 means each person with the virus passes it to one other person). Prior to schools reopening in the fall of 2020, these two groups were estimated to account for 75% of COVID-19 infections.[305]

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[302] Biswas et al., "Association of Sex, Age, and Comorbidities," 38.

[303] Pijls et al., "Demographic Risk Factors for COVID-19," 5–6 [and see p. 4, Table 2].

[304] Oster et al., "Transmission Dynamics by Age Group." The figures in column 3 won't add to 100% because each number is a percentage in that age group for those who tested positive on a RT-PCR test (such tests are discussed elsewhere in this volume).

[305] Monod et al., "Age Groups that Sustain Resurging COVID-19 Epidemics."

With each subsequent 'wave' of the virus studies have found increased risk for younger people. For example, a study reported in South Africa in late 2021 noted that in the wave of the pandemic occurring June to September, 2021, a significant increase had been seen in pediatric admissions to hospitals. In the subsequent wave, associated with the Omicron variant, children under age 18 were experiencing a 20% greater risk of hospitalization with a notable increase in admissions for those under age 5.[306]

Behavioral Factors

Behavior can produce risk in many situations, and the COVID-19 pandemic is one such. The following have been identified as *behavioral risk factors* (BRFs) increasing risk for severe outcomes:

- excessive alcohol consumption;
- physical inactivity;
- smoking; and
- unhealthy food intake.

In a study identifying BRFs like the above, 17,588 subjects from 23 European countries, plus Israel, provided data through a survey of health, aging, and retirement. More than a third (36%) engaged in at least one BRF, and about one-in-six (16.68%) engaged in three or more. The most common BRF was *physical inactivity* (48%), followed by *unhealthy food intake* (26%), *smoking* (18%), and *risky alcohol consumption* (7%). Coupled with these risky behaviors was also less adherence to known healthy behaviors against disease and its spread such as covering coughs and sneezes, hand-washing, wearing a mask, observing social distancing, and avoiding groups of more than five people. The researchers note that other studies also find that engaging in BRFs not only co-occur with each other (e.g., one might both smoke *and* drink excessively), but are also associated with other health risk factors such as less sleep. They found that "engagement in multiple BRFs increases the likelihood of noncompliance with preventive hygiene measures."[307]

Research suggests that *self-protective health behaviors (e.g., mask wearing, social distancing) are influenced by societal factors*. What one study termed 'a striking and robust pattern,' is that higher-income is strongly positively correlated to increases in self-protective behavior. Those in a low-income status are, for example, less likely to be able to work at home and more likely to be in jobs involving close contact with others (e.g., cashiers, packers and packagers). In response to the pandemic they found most people (88%) engaged in some self-protective behavior, but most low income people did not increase such behavior while high income people did.[308]

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[306] Discovery Health, "Discovery Health."
[307] Mendoza-Jiménez, Hannemann, and Atzendorf, "Behavioral Risk Factors." The quote is from p. 11.
[308] Papageorge et al., "Socio-Demographic Factors."

Blood type A has been identified as a risk factor for the severity of COVID-19. Specifically, research suggests two different blood groups having either lower or higher risk with COVID-19:

- *Type 0* is associated with *lower* risk;
- *Type A* is associated with *higher* risk.[309]

In the case of type A blood, the SARS-CoV-2 receptor-binding domain (RBD) of the spike (S) protein seems to prefer this blood type, but for reasons that remain murky. As we saw in an earlier chapter, the RBD has short, specific areas designed to facilitate *glycosylation*—a chemical reaction between the RBD and a receptor molecule of the host cell. The RBD glycosylation site secretes bits of RBD through glycosylation that then fold and bind to the host cell receptor. Blood groups A and B, through *glycotransferases* (catalytic enzymes involved in glycosylation), affect the glycosylation process in many cell types—including epithelial cells in the respiratory tract.[310] One suggestion is that the viral RBD may interact with blood group antigens, and with both SARS-CoV and SARS-CoV-2 the RBD appears to preferentially recognize the type of blood group A antigen uniquely expressed on respiratory epithelial cells.[311]

An interesting hypothesis set forward in one study suggests a *blood type-dependent infection pattern*. In this model, persons of the same blood type can infect each other with SARS-CoV-2, but may not always be able to infect people of a different blood type. Using the ABO group of blood types, the researchers used available datasets for COVID-19 infected people and stratified the information by blood type. After examining data in a comparative manner for select locations in six countries (China, Denmark, Italy, Spain, Turkey, and the U.S.), they then analyzed the early stages of the pandemic worldwide (78 countries).[312]

The researchers then formulated a hypothetical model predicated on the biology briefly explained above. SARS-CoV-2, like other viruses, depends on the host cell for glycan synthesis and modification. In short, for glycosylation to happen in the virus-host interaction, the virus needs the host cell to provide the glycan material that viral glycoproteins—vital to attachment and fusion—require. But this means that replicated virus particles take with them the glycan the host cell supplied—and this is the point where different blood types matter. For example, people with type O blood lack A or B antigens, so when a virus shows up with a glycan-motif derived from these other blood types, type O blood cells treat the newcomer as hostile. Its receptors refuse to recognize the unfamiliar glycan motif of the virus and thereby frustrate attachment and fusion. The host immune system also forms anti-A or anti-B antibodies.[313]

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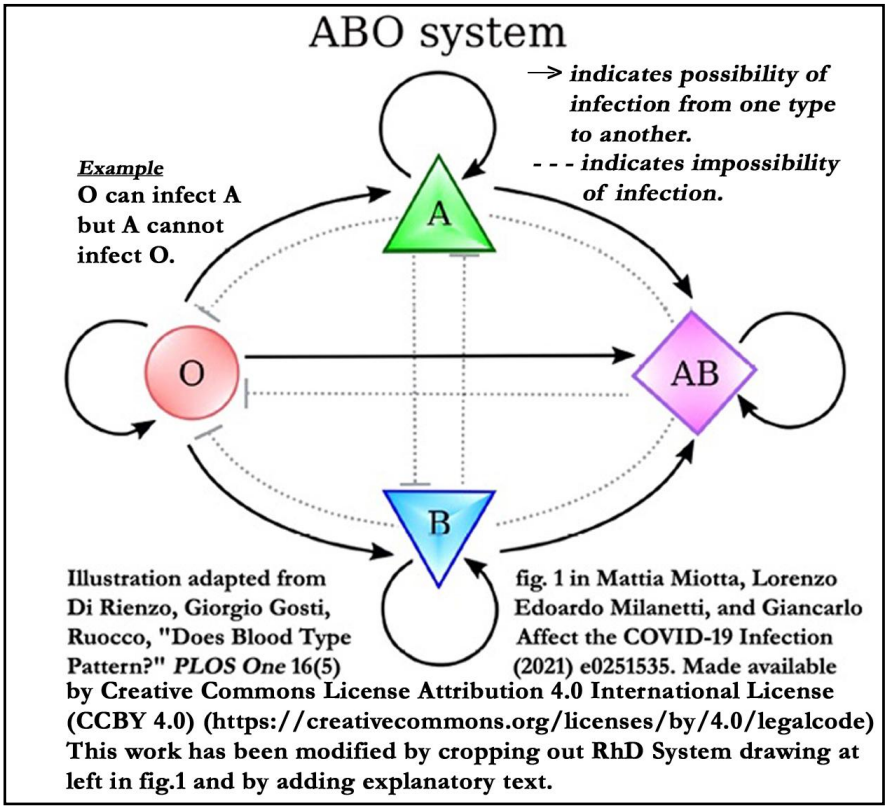
[309] The Severe COVID-19 GWAS Group, "Genomewide Association Study," 1531.
[310] Miotto et al., "Does Blood Type Affect the COVID-19 Pattern?" 2.
[311] Wu et al., "The SARS-CoV-2 Receptor-Binding Domain," 1308.
[312] Miotto et al., "Does Blood Type Affect the COVID-19 Pattern?" 3–14.
[313] Miotto et al., "Does Blood Type Affect the COVID-19 Pattern?" 15.

As a result, the researchers argue, certain 'rules' for the ABO group can be formed with respect to how they respond to each other when a viral particle displays a certain profile. These 'rules' are:

- O blood type forms antibodies to A and B blood types;
- A blood type forms antibodies to B blood type;
- B blood type forms antibodies to A blood type;
- AB blood type cannot form antibodies to the other types.

As they point out, these rules lead to the prediction that type O people should be underrepresented in the whole population of those infected by SARS-CoV-2—and that outcome actually has been observed. "Importantly," they continue, "these hypothesized asymmetrical transmission rules should affect the number of people with a certain blood type that can be potentially be infected because *they do not allow all infected to infect any potentially contacted person*."[314]

This model, which holds up well with existing epidemiological data, can be illustrated as follows:



[314] Miotto et al., "Does Blood Type Affect the COVID-19 Pattern?" 15 (emphasis added).

Increased body mass is associated with heightened risk for severity of COVID-19. *Body mass index* (BMI) is calculated for adults by dividing one's weight in pounds by the square of one's height, then multiplying the result by 703.[315] The Centers for Disease Control and Prevention (CDC) in the United States, and the World Health Organization (WHO) reckon a BMI of 25 or more as 'overweight,' with a BMI of 30 or more as 'obese.' A BMI of 40 or more is sometimes termed 'severe obesity.' Obesity is often associated with comorbid conditions—that is, it coexists alongside other health risks.

But even by itself, obesity constitutes a health risk. In COVID-19 cases obesity is associated with a greater risk for infection, hospitalization, and death.[316] In fact, one early retrospective study of 112 COVID-19 patients found that those with severe cases had significantly higher BMI (25.5) than a general group (BMI 22); among those who died of COVID-19, 88.2% had a BMI greater than 25, whereas among survivors only 18.9% did.[317]

One U.S. study examined patient data from 88 hospitals using the American Heart Association's (AHA) COVID-19 Cardiovascular Disease (CVD) registry through July 22, 2020. All patients age 18 or older with BMI data available were included—a total of 7,606 persons. Of this group, 30% were overweight and 43% were obese (21% Class I; 11% Class II; 11% Class III). The National Health and Nutrition Examination Survey (NHNES) was used for comparison; it reports more than 40% of adults age 50 or younger are obese, with 8% classified as severely obese (Class III). The hospital study found that patients with COVID-19 were more likely to be overweight or obese. In younger patients (age 50 or younger), with COVID-19, Class III obesity ('severe') was twice as common compared to the NHNES group. Overall, higher BMI categories for these patients were associated with four characteristics: [1] younger age; [2] female; [3] Black: and [4] diabetes comorbidity. Hospital outcomes were less favorable to those with higher BMIs. Obesity was associated with a higher risk for mechanical ventilation and death.[318]

The search for an underlying biological mechanism for this increased risk has implicated SARS-CoV-2 infection in human adipose tissue (body fat). One obvious issue is that the receptor the virus attaches to—hACE2—is common in adipose tissue. In fact, it has higher expression in adipose tissue than in lung tissue.[319] Research reported late in 2021 found evidence that adipose tissue supports viral infection that elicits an inflammatory response by the body. The

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[315] Or one can use *Calculate Your Body Mass* at https://www.nhlbi.nih.gov/health/educational/lose_wt/BMI/bmicalc.htm.

[316] Kompaniyets et al., "Underlying Medical Conditions," 3; also Martínez-Colón et al., "SARS-CoV-2 Infects Human Adipose Tissue," 4.

[317] Kassir, "Risk of COVID-19 for Patients with Obesity."

[318] Hendren et al., "Association of Body Mass Index and Age with Morbidity and Mortality."

[319] Kassir, "Risk of COVID-19 for Patients with Obesity," citing Jia et al., "Two Things."

study looked at multiple sites ('depots') of adipose tissue and found infected *adipocytes* ('fat' cells) and *adipose tissue macrophages* (ATMs; macrophages are a kind of leukocyte important in body inflammatory response). The macrophages showed the most infection and the infected macrophages displayed an increase ('upregulation') of inflammatory mechanisms. This inflammatory response might contribute to severity of disease as seen in obese patients.[320]

Vishwa Deep Dixit, a Yale School of Medicine professor of immunology, has mused, "Maybe that's the Achilles' heel that the virus utilizes to evade our protective immune responses — by hiding in this place."[321]

Environment

'Environment' here means physical spaces and their conditions (e.g., temperature and humidity), the people inhabiting those spaces, and what they are doing. There are reasons for recommendations and mandates such as that termed *social distancing*. As we saw earlier in the chapter, SAR-CoV-2 is spread through the air, passing from an infected person to an uninfected one via aerosols and droplets passed from the former to the latter. This means the distance virus-carrying particles can travel matters. That conclusion means at least two other factors must be considered:

- some environments are inherently more risky; and
- some people are better spreaders of the virus than others.

After some two years of living with COVID-19 it has become obvious that the virus is capable of spreading all year long no matter the climate or season. However, it spreads more readily in cold weather. That is because cold weather drives people indoors and *where people congregate in enclosed spaces they are more at risk of infection*.

Because the virus can survive outside a host for a modest period of time, any space that has recently been occupied by an infected person might leave viral particles even after the infected person is gone. That is why so many places have taken precautionary steps to disinfect surfaces regularly.

The second factor named above is that not all people are equal when it comes to spreading SARS-CoV-2. But before we discuss that further a seemingly obvious point that nevertheless has evaded comprehension by some folk needs to be emphasized: *no one can be infected if no infection is present, but no one can be sure the people one is around are uninfected*. Some infected people show no symptoms at all—they are *asymptomatic*. Those that are infected typically display no symptoms at the start of their infectious period and this at the very time when they may be most infectious. These matters are discussed further below.

Environments are more than just spaces. They become matters of concern when they are occupied, and the greater the occupancy, the closer people are crowded together, and the more they are breathing on each other the higher the

[320] Martínez-Colón et al., "SARS-CoV-2 Infects Human Adipose Tissue."
[321] Dixit quoted in Rabin, "The Coronavirus Attacks Fat Tissue."

risk of infection from whatever airborne infectious agents are present. With respect to COVID-19 this reality was forcefully driven home by what have become known as *superspreader events*. Perhaps the first to capture widespread attention was early in the pandemic, in March, 2020, when infection spread among members of choir who had gathered for a 2½ hour choir practice. Of the 61 who attended, 53 (87%) became infected, with 3 (6% of the infected) being hospitalized, and 2 (4% of the infected) dying.[322]

Research has demonstrated conclusively some important matters about people who can spread COVID-19 more readily. Consider these findings from study of producing 'respiratory aerosol emissions' (i.e., breath released as a spray of fine particles):

- *activities where breath is forcefully expelled* (e.g., singing) more readily spread SARS-CoV-2;
- *adults produce more aerosol* than minors do;
- *males produce more aerosol* than females do; and,
- *some individuals may become 'superspreaders'* due to a combination of their individual physiology with other factors.

A study published late in 2021 recruited healthy volunteers to measure respiratory aerosol emission to determine such factors as those named above. The volunteers were evenly split between minors (ages 12–17) and adults (ages 18–64). However, they also were participants from four performance specialties (actors, dancers, vocalists, and woodwind/brass instrumentalists). Although they were asked to perform certain acts related to their specialty and ability level, what the study was interested in was their performance on two generic vocal tasks: singing ('Happy Birthday') and talking (reading aloud a passage titled 'The Caterpillar'). Their global findings were that, on average, singing produced 77% more aerosol than talking, that adults produced 62% more aerosol than minors, and that males produced 34% more aerosol than females. Interestingly, they found that the aerosol emissions from the highest emitting singer were *500% higher* than the median value, and more than 42 times higher than the lowest emitting singer. The researchers suggest that such a person if infected and behaving in activities and patterns that include more contacts with others might become a superspreader of the virus.[323]

In sum, environmental factors include both the space one is within and the people one shares that space with, and what those people are doing.

Ethnicity and Race

Facts are stubborn things and as much as many Americans are discomfited by the reality of systemic racism, it exists and it complicates many areas of life, including matters related to the COVID-19 pandemic. Research has shown that

[322] Hamner et al., "High SARS-CoV-2 Attack Rate."
[323] Good et al., "Respiratory Aerosol Emissions from Vocalization."

whether one looks at large studies or smaller ones, whether those studies restrict themselves to specific places or look at a larger picture, similar and dismaying evidence emerges.

For instance, a study reported in *The New England Journal of Medicine* in 2020 examined 3,481 positive COVID-19 cases in a health care system in Louisiana. Although Black Americans make up less than a third (31%) of those in the health system, they constituted more than two-thirds of those who tested positive for COVID-19. Of this group, 1382 patients (39.7%) were hospitalized. Black patients constituted more than three-quarters (76.9%) of the hospitalized. They also were those most likely to die (70.6%).[324]

A much larger scale study examined 369 counties in the United States with a total population of 102,178,177 people. These counties were spread across seven states (California, Louisiana, Massachusetts, Michigan, New Jersey, New York, and Pennsylvania) in diverse parts of the nation, but linked by high infection rates of SARS-CoV-2. That study highlighted that race—especially being Black—was a risk factor for infection. The number of African American infected cases per million people compared to White infected cases was a little more than *3 times greater*.[325] Another study summed up the situation in the U.S. this way: "Among >1.75 million persons with SARS-CoV-2 in 23 US jurisdictions during January 1–October 1, 2020, persons from most racial/ethnic minority groups had higher cumulative incidence than White persons."[326]

Of course, it is not just the United States where unequal health outcomes are associated with race or ethnicity. A study in Canada concluded, "Similar to the U.S. and U.K., urban regions in Canada with higher shares of Black residents have been disproportionately impacted by COVID-19."[327]

Other ethnic/racial groups may also be at risk. One study estimated that in the early pandemic period (through 2020) life expectancy at birth decreased by 3.25 years (4.4% of expected life span) for Blacks—a decrease 2.4 times higher than for Whites. The decrease in life expectancy was even greater among Hispanic people—3.88 years (4.7%), or 2.9 times higher than for Whites.[328] In late 2021, the CDC reported, "Based on mortality data from CDC's National Vital Statistics System (NVSS), from February 1, 2020 to September 30, 2021, there have been an estimated 700,000 excess deaths in the United States. The largest percentage increases in mortality occurred among adults aged 25–44 years and among Hispanic or Latino people."[329]

(Ethnic/race factors are closely tied to societal factors examined later.)

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[324] Price-Haywood et al., "Hospitalization and Mortality." The authors note (p. 2541) that after adjustment for differences in sociodemographic and clinical characteristics at admission the simple fact of one's race is not associated with higher in-hospital mortality.

[325] Abedi et al., "Racial, Economic, and Health Inequity in COVID-19 Infection," 740–41. The actual numbers (p. 732): 1,981 African-American cases/million to 658 White cases/million.

[326] Hollis et al., "Racial and Ethic Disparities in Incidence of SARS-CoV-2."

[327] Choi et al., "Studying the Social Determinants of COVID-19," 159.

[328] Woolf, Masters, and Aron, "Effect of the Covid-19 Pandemic," 3.

[329] CDC, "Underlying Medical Conditions."

Genetic Factors

The role individual genetic makeup may play also has received attention. For example, a study reported by The Severe COVID-19 GWAS Group, involving 1,980 patients in a genome wide study, identified a gene cluster at chromosome 3p21.31 as a genetic susceptibility locus in patients experiencing respiratory failure while with COVID-19.[330]

One meta-analysis, drawing upon 46 studies across 19 countries and involving nearly 50,000 COVID-19 patients found 13 independent places along the human genome—called *genome-wide specific loci*—to be of particular importance. Of these, six had association with critical COVID-19 illness and nine with moderate to severe (including 5 of the 6 for critical illness). None of these were sex-specific. Some of these correlate to lung, autoimmune and inflammatory diseases. One—a genetic liability to ischemic stroke—was related to a greater likelihood of hospitalization, but not to a greater likelihood of infection. The study also found that increased risk of severe COVID-19 is associated with genetically predicted higher body mass index (BMI) and smoking; the former is also associated with increased risk of infection.[331] All of this kind of research is ongoing but it has potentially high value for both risk assessment and treatment.

Geography

Though not much has been made of it, some research has suggested increased vulnerability for those residing in northern latitudes. For example, a study released in Spring, 2020, looking at 117 countries, reported an estimated 4.4% increased mortality rate per degree of latitude north of 28 parallel north[332]—a latitude crossing the top of Mexico, the African continent, Saudi Arabia, and India. Thus all of the United States is above this latitude; the 40th parallel north, for example, crosses through Boulder, Colorado and Philadelphia, Pennsylvania.

Geography is closely tied to *weather*, which we saw near the start of this chapter plays a role in transmission of SARS-CoV-2. We noted then the factors of temperature and humidity emerged as the two most significant weather factors. But they always occur in some geographical context. For example, as one study noted, where relative humidity proved positively correlated with viral transmission in Singapore and Brazil, it did not in New York and India. An increase in relative humidity might help spread the virus in arid inland cities but a decrease in the same might help spread it in coastal cities. The researchers conclude that the relationship between COVID-19 transmission and meteorological factors is complex and varies among different geographical regions.[333]

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[330] The Severe COVID-19 GWAS Group, "Genomewide Association Study," 1529 (identifying 6 genes: SLC6A20, LTFL1, CCR9, FYCO1, CXCR6, and XCR1).

[331] COVID-19 Host Genetics Initiative, "Mapping the Human Genetic Architecture of COVID-19."

[332] Rhodes et al., "COVID-19 Mortality Rate Increases with Northerly Latitude."

[333] Yang, Li, and Cao, "Influence of Meterological Factors."

Pregnancy as a risk factor for developing severe COVID-19 met with mixed conclusions in early research. One report examined a range of literature on the subject and concluded that pregnant women were similar to others and at no higher risk for developing critical pneumonia. However, infection might result in problems for their babies such as preterm labor or fetal distress.[334] On the other hand, a different review of the evidence concluded that pregnant women are at increased risk of severe disease, with greater likelihood of hospitalization, ICU admission, and mechanical ventilation.[335]

On the whole, research suggests *pregnancy increases risk of severe COVID-19*. A study covering the period January 22–October 3, 2020, examined data on 409,462 women of childbearing age (15–44), all of whom had symptomatic COVID-19. Of this group, 23,434 (5.7%) were pregnant. After adjusting for age, race/ethnicity, and underlying medical conditions, pregnant women were significantly more likely to experience the following events:

Pregnant Women compared to Non-Pregnant Women	**Adjusted Risk Ratio (x Greater Risk)**
admission to Intensive Care Unit (ICU)	3.0 (3-fold)
receive invasive ventilation	2.9 (2.9 times)
receive extracorporeal membrane oxygenation (ECMO)	2.4 (2.4 times)
die	1.7 (70% greater)[336]

One meta-analysis of the topic whittled 429 studies down to 27 studies, involving seven countries (in order of number of studies: China, U.S., France, Italy, United Kingdom, Spain, and Iran). The result was a subject population of 745 pregnant women with confirmed infections by SARS-CoV-2 and 219 'control' subjects. To these were added 45 cases from 34 case reports from 15 countries. The average maternal age was 31 years old. In addition to the adult women, 505 neonates were included. Here are some of the results:

COVID-19 Symptoms	**% cases**	**Pregnancy Outcomes (Successful Births)**	**% cases**
fever	58%	*overall for an adverse event*	27%
cough	52%	preterm birth	23%
lymphopenia *(low white blood cell count—i.e., those with lymphocytes)*	46%	low birthweight	7%
dyspnea *(labored breathing)*	17%	infected newborn	1%
asymptomatic (no symptoms)	9%		

[334] Dana et al., "COVID-19 and Pregnancy."
[335] Kucirka, Norton, and Sheffield, "Severity of COVID-19 in Pregnancy."
[336] Zambrano et al., "Update: Characteristics of Symptomatic Women."

The risk for a preterm birth was 2.28 times greater.[337]

A comprehensive analysis noted the increased risk for pregnant women and also drew attention to complications specific to the unborn child. Specifically, they examined evidence for transmission of SARS-CoV-2 from mother to fetus—*vertical transmission*—and reported studies finding virus in the placenta and elevated inflammatory cytokines (showing immune system reaction to infection) in the serum of neonates at delivery.[338]

Psychological Factors

Stress has been mentioned often with respect to COVID-19. The long duration of the pandemic has brought psychological stress upon most people. It is normal to experience anxiety when confronted by an unknown situation where the threat of harm is suggested. But typically anxiety, or the fear that may arise, is limited in duration. A threat is identified, assessed, and responded to in some manner. But COVID-19 has kept levels of anxiety elevated for many people for a very long time. Ilene Weingarten, a therapist who has witnessed among her clients the effects of this prolonged stress, observes that ordinarily a stressful or traumatic situation is resolved and its impact fades over time. But the pandemic, she notes, has been "relentless." As a result people are at risk of getting stuck, with the anxiety producing continuous reaction to it that, over time, narrows one's view of the world, robbing things of context, and taking a toll on mental health. Even shortly after the rise of the Omicron variant she observed that with it "there's been a spike in disheartened feelings, feelings of hopelessness and helplessness."[339]

Realistic anxiety motivates problem-solving behavior, such as being vaccinated, wearing a mask, and observing social distancing. Unrealistic anxiety motivates denial, flights of fantasy, and other unproductive, dangerous behavior. When normal anxiety goes awry, psychological disorder may result. *Anxiety and fear-related disorders* have emerged as a major risk factor for death among those severely ill with COVID-19 (a matter discussed late in the chapter).[340]

Early on one concern for older adults as a particularly vulnerable group was that they would experience more stress and anxiety. However, a study of 515 adults ages 20–79 concerning anxiety over developing COVID-19 found no age differences in stress levels. Older adults showed more knowledge about COVID-19 and were more likely to endorse proactive coping (e.g., covering nose and mouth when coughing or sneezing, avoiding gatherings, disinfecting surfaces). The authors noted, "The interactions between age and proactive coping and age and anxiety about developing COVID-19 highlight the notion that there is a

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[337] Statistics and table data derived from Dubey et al., "Maternal and Neonatal Characteristics" (see. p. 394 for data used in table).

[338] Moore and Suthar, "Comprehensive Analysis of COVID-19 during Pregnancy," 181–82.

[339] Weingarten, quoted in Bahrampour, "They Were So Careful."

[340] Kompaniyets et al., "Underlying Medical Conditions," 5.

diversity of experiences within older adults and that not all older adults respond the same way to the same stressor."[341]

Sex

From very early in the study of COVID-19 it was observed that men were at more risk of dying from the disease than women.[342] Specific results vary somewhat across studies as to the magnitude of difference but the general conclusion that males are more vulnerable to the disease has been consistent. Here we examine only a few of the studies available.

The meta-analysis of 59 studies comprising 36,470 patients in 8 countries referred to earlier found that compared to women, men had a higher relative risk of infection (8% higher), developing severe illness (18% higher), being admitted to an Intensive Care Unit, and dying from COVID-19.[343] Another study—a retrospective cohort study of hospital patients from April–June, 2020—looked at 66,646 admissions (across 613 hospitals) with a COVID-19 diagnosis. These admissions resulted in 12,388 hospital deaths (18.6%). In looking at a multitude of factors (a 'multifactorial analysis'), males were found to be at 30% higher mortality risk.[344] Another meta-analysis, following a systematic review of the literature, examined 20 studies with a total of 64,676 COVID-19 patients. After pooled risk analysis, it found male patients with an 86% greater chance of death (Relative Risk (RR) 1.86), despite only having a 28% greater risk of infection.[345] In other words, though males are at greater risk of infection by SARS-CoV-2, once infected they are at far greater risk of death.

There may be a variety of contributing reasons for this, ranging from biological factors to behavioral ones. Another study looked at 10 European regions (Denmark, Norway, Sweden, The Netherlands, England and Wales, France, Germany, Italy, Spain and Portugal) for a period between February 14–June 29, 2020. The relative risk (RR) of death for men was higher in all age groups (except for those under age 60 or older than age 80 in Norway, and those under age 60 in Denmark). The risk of death increased with age, but the smallest difference in mortality was found after age 80.[346]

One possible biological factor is related to the *normal decrease in testosterone levels* as men age. As these levels decline, the level of hACE2 increases. This increase contrasts with lower levels of hACE2 activity found in younger healthy individuals.[347] As we have seen in previous chapters, hACE2 is the receptor on cells that SARS-CoV-2 binds to as it attaches and enters human host cells.

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[341] Pearman et al., "Age Differences in Risk and Resilience." Quote is from p. 5.
[342] Liu et al., "Risk Factors," 4, notes males of all ages are more at risk for severe COVID-19.
[343] Pijls et al., "Demographic Risk Factors for COVID-19," 4–5 [and see Table 2].
[344] Goodman et al., "Impact of Sex," e4118.
[345] Biswas et al., "Association of Sex, Age, and Comorbidities," 38.
[346] Ahrenfeldt et al., "Sex and Age Differences in COVID-19 Mortality in Europe."
[347] Singh et al., "Gender and Age Differences Reveal Risk Patterns," 55.

A likely factor is *differences between males and females in immune system functioning*. The observed sex differences in human immune system functioning and age-related changes remain poorly understood. Females typically show stronger immune responses than do males. This quality, however, appears to be a two-edged sword: on the one hand, it means their systems clear pathogens more rapidly and they respond better to vaccines, yet on the other hand they are more vulnerable to auto-immune diseases (80% of such cases are female).[348]

Another possible factor is the *tendency of men to have more life-threatening comorbidities* (e.g., heart disease). Another possible factor is *differences in behavioral choices*. For example, *smoking* increases risk of severe symptoms of COVID-19 (1.4x more likely for smokers), and the need for mechanical ventilation (2.4x greater risk).[349]

At least some of the observed differences may be due in part to the social roles men more often occupy than women, but social roles, occupations, and behavioral choices all make comparisons of the sexes a complicated endeavor. In the end, whatever may explain the differences in COVID-19 experiences between men and women remains unclear, though the general findings remain relatively consistent across studies.

Societal Factors

Societal factors that increase risk are especially associated with particular groups characterized by ethnicity, race, or social class. Thus, for example, the poor (a social class) are less likely to enjoy protections the rest of us take for granted. When infected they are also less likely to have resources to use medical or other assistance.

Inequities in societal factors related to health and well-being are implicated in an increased risk of severe illness from COVID-19. Such factors include:

- lack of access to health care;
- lack of economic stability; and,
- lack of education.[350]

Clearly the operative word here is *lack*. Things many Americans take for granted are absent for a significant number of people. *Lack of access to health care* means preventive screenings, ordinary preventive measures (e.g. flu shots), and managed care by a primary care physician (PCP) may be lost, and medical care routinely available to the insured is denied. This factor is closely associated with the next. *Lack of economic stability* often means instability in other life areas, such as homelessness, frequent relocation, and so forth. In terms of health care, as just noted, it generally means lack of health insurance, lack of a designated PCP to oversee and assist health choices, and a sense of few options in coping with

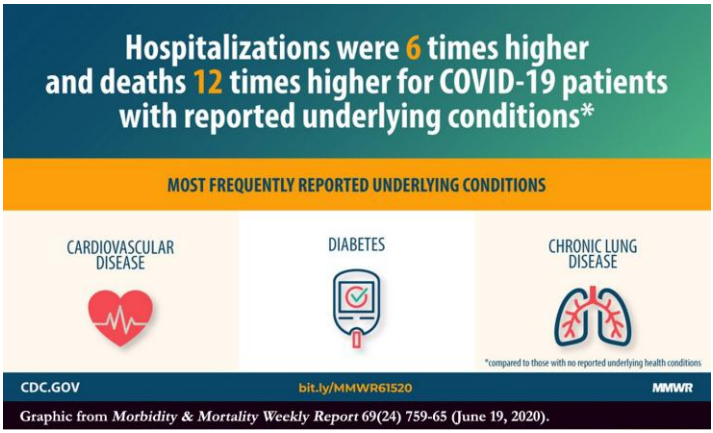
[348] Márquez et al. "Sexual-Dimorphism in Human Immune System."
[349] Ahrenfeldt et al., "Sex and Age Differences in COVID-19 Mortality in Europe," 396.
[350] For more on these factors and their correlation to issues of ethnicity and race, see CDC, "Risk of Severe Illness."

health risks. *Lack of education* is associated with diminished understanding of available resources and options and greater vulnerability to misinformation.

One study examined all 50 states and the District of Columbia (Washington, D.C.), but for a restricted span—March 25–July 29, 2020 (weeks 9 to 27 of the pandemic). At the conclusion of this period there were 4,289,283 COVID-19 cases with 147,074 deaths. Using a cross-sectional analysis the researchers found a significant association between COVID-19 incidence-and-mortality and social risk factors (using the Social Vulnerability Index (SVI)). A small increase in SVI correlated to a large one in incidence of infection or mortality. Thus, for example, an increase of 0.1 point SVI correlated to a 14.3% increase in infection numbers and 13.7% increase in mortality rate. Like other studies, they found a close association between minority ethnic/racial status and deprivation in key social factors. For example, low income minorities often work in essential worker occupations interfacing with the public, while living in crowded housing and relying on public transportation—all factors contributing to increased exposure and transmission of the disease.[351]

Remember, societal factors like those mentioned here are also strongly correlated with ethnicity and race factors. Put most bluntly, despite the resistance of many Whites to acknowledge systemic racism it is a fact with significant health implications. Alongside race, the large scale (369 counties in 7 states embracing 102,178,177 people) study mentioned earlier found that for COVID-19 social risk factors for increased mortality included poverty, lower education, and lack of insurance.[352] The United States, of course, is not alone in experiencing the adverse effects of ethnicity/race and social factors colluding to hurt minority communities. A study in Canada, for example, also found race and poverty linked with COVID-19 infections.[353]

Underlying Medical Conditions



Graphic from *Morbidity & Mortality Weekly Report* 69(24) 759-65 (June 19, 2020).

[351] Karmakar, Lantz, and Tipirneni, "Association of Social and Demographic Factors."
[352] Abedi et al., "Racial, Economic, and Health Inequity in COVID-19 Infection," 738.
[353] Choi et al., "Studying the Social Determinants of COVID-19."

Decreased immunological protection also occurs among those who are immunocompromised for one reason or another or who have one or more underlying medical conditions. Underlying conditions that are associated with severe illnesses (as indicated by a scientific measure such as meta-analysis or systemic review), as listed by the CDC in late 2021, include (in alphabetical order):

- Cancer
- Cerebrovascular disease
- Chronic kidney disease
- Chronic lung diseases:
    - interstitial lung disease
    - pulmonary embolism
    - pulmonary hypertension
    - bronchopulmonary dysplasia
    - bronchiectasis
    - chronic obstructive pulmonary disease (COPD)
- Chronic liver diseases:
    - cirrhosis of the liver
    - non-alcoholic fatty liver disease
    - alcoholic liver disease
    - autoimmune hepatitis
- Diabetes mellitus (Type 1 & Type 2)
- Heart conditions:
- Mental health disorders:
    - mood disorders (including depression)
    - schizophrenia spectrum disorders
- Pregnancy (or recent pregnancy)
- Smoking (current or former)
- Tuberculosis[354]

Any underlying medical condition can complicate a person's situation when ill with COVID-19, but the above are those best authenticated at increasing risk of a severe illness. In general, a serious medical condition weakens a person making them more vulnerable to infections. With respect to COVID-19, we might suspect and in fact find that the conditions listed are also target areas for SARS-CoV-2. Put simply, places in the body where the hACE2 receptor the virus uses to attach and enter host cells are likely to experience disease effects from the viral infection. Such places in the body include the lungs—which gives rise to seeing COVID-19 as a respiratory disease—but hACE2 expression may be even higher in the heart, kidney, testes, intestine, bladder, gallbladder, and

[354] CDC, "Underlying Medical Conditions." Also see Liu et al., "Risk Factors," 4.

adipose ('fat') tissue.[355] But these places might also be the site of medical conditions *before* virus infection because other diseases are also correlated with hACE2 activity. For example, essential hypertension and cardiovascular disease both have been linked to hACE2 levels of activity.[356] Hypertension, one meta-analysis reported, results in a 95% (RR 1.95) greater relative risk of mortality.

Here are relative risks associated with various comorbidities as reported in a systematic review and meta-analysis involving 64,676 COVID-19 patients:[357]

Condition	Relative Risk (% greater risk)	Condition	Relative Risk (% greater risk)
cancer	RR 1.89 (89%)	*hypertension*	RR 1.95 (95%)
cardiovascular disease	RR 3.05 (3.05-fold)	*kidney disease*	RR 4.90 (4.90-fold)
cerebrovascular disease	RR 4.78 (4.78-fold)	*liver disease*	RR 1.64 (64%)
diabetes	RR 1.97 (97%)	*respiratory disease*	RR 2.74 (2.74-fold)

It needs to be underscored here that many more underlying conditions than those mentioned here might complicate the experience of COVID-19. The virus weakens and sometimes kills the cells it infests. Because these are widespread through the body when other conditions also exist the damage might be worse.

Vaccination Status

Vaccination status might rank as the most significant individual factor in assessing infection risk—and the one most completely under individual control. The evidence is unambiguous—vaccines offer a significant level of protection that anti-vaxxers deny themselves, to their own harm and that of others.

Even with presumed reduced effectiveness of the vaccines against the Omicron variant, the vaccinated remain at less risk than the unvaccinated. The United Kingdom Health Security Agency (UKHSA) reported *risk of hospitalization for the fully vaccinated as 65% lower than the unvaccinated—and for those who had the booster shot it was 81% lower*.[358] Similar findings were reported in Ontario, where the hospitalization rate per million was 5 times higher for the unvaccinated—or put in terms of relative risk, the vaccinated are at 80.1% less risk.[359] The *New York Times* early in 2022 reported that data from New York City indicated that the previously seen gap between vaccinated and unvaccinated was even more pronounced with Omicron, with the unvaccinated more likely to be hospitalized and to require care in an Intensive Care Unit (ICU).[360]

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[355] Jia et al., "Two Things to Know."

[356] Singh et al., "Gender and Age Differences Reveal Risk Patterns," 55. I have added the material in parentheses because many folk are not familar with relative risk (or 'risk ratio') reporting and how to translate it into the more ordinary way people talk. Thus RR 4.90 means 4.9 times greater relative risk (or 4.9-fold greater relative risk). Relative risk is used to compare the odds of two groups against each other—in this case the odds for males as opposed to the odds for females.

[357] Biswas et al., "Association of Sex, Age, and Comorbidities," 40.

[358] UK Health Security Agency, "SARS-CoV-2 Variants" (Technical Briefing: Update), 5.

[359] Bailey, "Omicron vs. the Unvaccinated and the Vaccinated."

[360] Leatherby and Lutz, "Early Data Hints at Omicron's Potential Toll Across America."

The following graph illustrates the contrast in outcomes between the vaccinated and unvaccinated over a specified period (Apr. 10–Oct. 30, 2021):

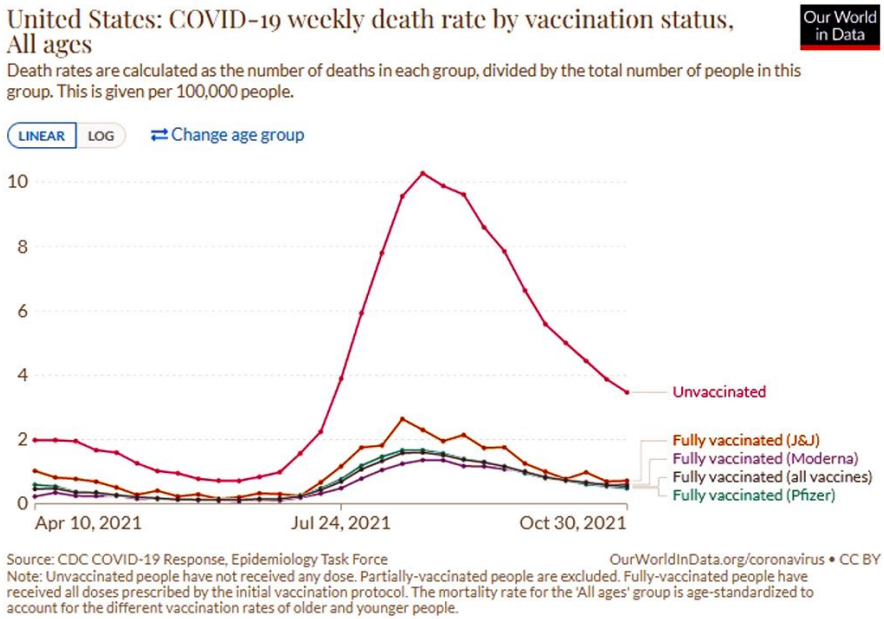


Illustration courtesy of *Our World in Data* (https://ourworldindata.org/covid-deaths-by-vaccination), as embedded in Edouard Mathieu and Max Roser, "How Do Death Rates from COVID-19 Differ between People Who Are Vaccinated and Those Who Are Not?" (Dec. 27, 2021 update). The material is made available by Creative Commons BY License - CC-BY (https://creativecommons.org/licenses/by/4.0).

It is easy to see that the pandemic's effects varied at different times. The illustration above shows the advent of Delta in the U.S., which peaked in September, and began to decline in October. It is also easy to see that *at all times the vaccinated fared better than the unvaccinated.*

A study released in my home state of Washington at the end of 2021 was titled, "COVID-19 Cases, Hospitalizations, and Deaths by Vaccination Status." Its summary of results, highlighting the relative risk faced by the unvaccinated at the end of that year, can be tabulated as follows:

Age Range	Comparative Risk of Infection	Comparative Risk of Hospitalization
12–34 years old	*3x* more than vaccinated	*12x* more than vaccinated
35–64 years old	*4x* more than vaccinated	*18x* more than vaccinated
65 years old and older	*7x* more than vaccinated	*13x* more than vaccinated (*15x* risk of death)[361]

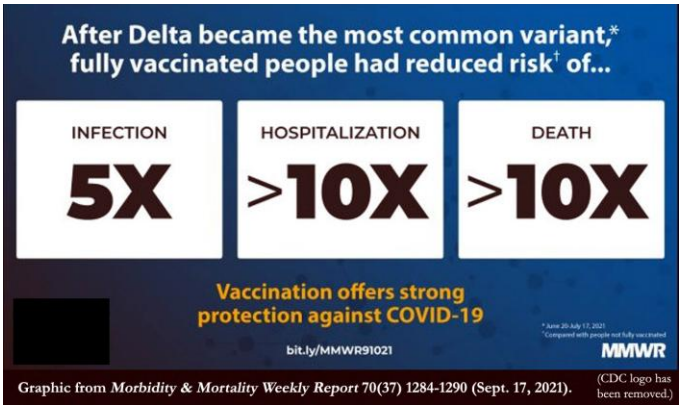
'Vaccinated' means *fully* vaccinated—1 shot of Johnson & Johnson vaccine, or 2 shots of Pfizer or Moderna vaccines—plus at least 2 weeks time after last shot.

[361] Washington State Dept. of Health, "COVID-19 Cases, Hospitalizations, and Deaths," 5–10.

The report found that for the period February 1–December 21, 2021, for those age 12 and older, the unvaccinated accounted for *75% of all reported cases of COVID-19*. For the slightly shorter period of February 1–December 14, 2021, the unvaccinated accounted for *80% of all reported cases of hospitalizations* because of COVID-19. For the period February 1–November 30, 2021, the unvaccinated accounted for *75.3% of all reported cases of death* because of COVID-19—and this despite constituting just over one-fifth of the State's population (aged 12 and older).[362] Anyway it is looked at, the unvaccinated account for a highly disproportionate amount of cases of infection, hospitalization, and death.

The numbers are particularly dramatic among the young. Among children ages 12–17, 89% of those who contracted COVID-19 through the end of November were unvaccinated though they accounted for only a little more than a third (35.1%) of that age group.[363] Put differently, about one-third of children were bearing about 90% of COVID-19 cases because they were unvaccinated—a choice made by their caretakers, not themselves. (How this can be construed as ethical, responsible parenting eludes me.) Although the unvaccinated young, ages 12–34, accounted for only a little more than a quarter (27.3%) of that age group, they accounted for 92.1% of hospitalizations in that age group.[364]

One belief held by the anti-vaccine movement is that vaccination is itself dangerous and can result in death. Anti-vaxxers often draw a picture of a vaccine posing more of a health threat than COVID-19. But do the vaccinated stand at more risk of death from non-COVID-19 causes because they are vaccinated? A major study addressed that very question. The cohort study, conducted December 20, 2020–July, 2021, involved approximately 11 million people (6.4 million vaccinated; 4.6 million unvaccinated) enrolled in seven Vaccine Safety Datalink (VSD) sites. It found that not only was there no increased risk for mortality among the vaccinated, but that after standardizing mortality rates by age and sex (done to get the most accurate overall picture), *COVID-19 vaccine recipients had lower non-COVID-19 mortality than did the unvaccinated.*[365]



Graphic from *Morbidity & Mortality Weekly Report* 70(37) 1284-1290 (Sept. 17, 2021). (CDC logo has been removed.)

[362] Washington State Dept. of Health, "COVID-19 Cases, Hospitalizations, and Deaths," 11.
[363] Washington State Dept. of Health, "COVID-19 Cases, Hospitalizations, and Deaths," 12.
[364] Washington State Dept. of Health, "COVID-19 Cases, Hospitalizations, and Deaths," 13.
[365] Xu et al., "COVID-19 Vaccination and Non-COVID-19 Mortality Risk."

The role of vitamin D in immune system support is discussed in a following chapter. However, here we need to note that various studies have found an association between vitamin D deficiency and increased severity of COVID-19. This may be linked to the geographical factors discussed above in that there is decreased absorption of vitamin D from sunlight (ultraviolet radiation) in northern climes in autumn and winter.[366]

A systematic review and meta-analysis of the scientific literature published until June 8, 2020, reduced 1195 studies screened for the review's eligibility criteria to 6 retrospective studies (3 European; 2 Asian; 1 American). The result was a pool of 1,368 COVID-19 patients (58.6% male; mean age 63.8 years old). The researchers found that patients with a poor prognosis for COVID-19 had significantly lower levels of Vitamin D compared to those patients receiving a better prognosis.[367]

An even larger study reviewed 54 reports covering 1,403,715 people in 8 different regions of the world (Central America, Central Asia, Europe, Far East Asia, Middle East, North Africa, North America, and Southeast Asia). Among its results (measuring serum 25-hydroxyvitamin D (250HD) levels) were the following:

Variable	**Sample Size Considered**	**Vitamin D Status (by nmol/L)**	**Increased Risk (Pooled Odds Ratio)**
Positive vs. Negative Cases (Infection)*	26,333	insufficiency[1]	1.49 (49% greater)
	380,357	deficiency[2]	1.83 (83% greater)
	1,383,867	severe deficiency[3]	1.68 (68% greater)
Hospitalization*	1,370	insufficiency	1.82 (82% greater)
	1111	deficiency	2.38 (2.38x greater)
	1670	severe deficiency	2.51 (2.51x greater)
ICU Admission*	1,745	insufficiency	2.83 (2.83x greater)
	2,611	deficiency	2.16 (2.16x greater)
	1,547	severe deficiency	2.63 (2.63x greater)
Mortality	1,676	insufficiency	4.15 (4.15x greater)
	4,364	deficiency	1.84 (84% greater)
	4,265	severe deficiency	2.60 (2.6x greater) [368]

*Reporting whole available datasets. [1]<75 nmol/L [2]<50 nmol/L [3]<25 nmol/L (Thresholds)

[366] Rhodes et al., "Perspective: Vitamin D Deficiency," 98–99.
[367] Munshi et al., "Vitamin D Insufficiency."
[368] Chiodina et al., "Vitamin D Status and SARS-CoV-2 Infection."

As the above results suggest, even a modest lack of vitamin D can increase risk of infection and severe outcomes.

Special Note #1: Influence of Multiple Factors

While it may be obvious it is important to formally acknowledge that individual factors often combine to increase health risk. Just as in medical circles there is widespread acknowledgment of the influence of *comorbidities*—concurrent medical conditions (e.g., obesity and diabetes)—so in broader perspective coexisting cofactors can negatively influence health risk. Sometimes studies point this out explicitly. For example, in the United Kingdom a population-based cohort study of 473,550 volunteers (all older than age 50) examined risk factors for dying from COVID-19. From the start of February, 2020 until September 21, 2020, there were 459 COVID-19 (and 2626 non-COVID-19 deaths). The study found age, sex, and race/ethnicity to be the three factors most strongly correlated with COVID-19 death. Specifically, the deceased were older (6.6 years mean age greater), and more likely to be male and Black. In addition, the research also reported there was a higher risk for those with less education and low earnings, among other social factors. Medical comorbidities also increased risk of death. In short, this larger study found many of the factors listed above to apply to the group who died of COVID-19.[369] Of course, not every individual has every factor that increases risk, but it seems sensible to acknowledge that increasing the number of factors influences the degree of cumulative risk.

Special Note #2: A Potential Modifying Factor

A population-based longitudinal study was conducted in the United Kingdom from May 1, 2020 to February 5, 2021, involving 15,227 participants (69.8% female; 94.9% White). Of these, 446 persons tested positive for COVID-19, of whom 32 required hospitalization. The study examined a number of variables to calculate associations between each variable and risk of COVID-19. They found a number of associations heightening risk such as some of those discussed above. But they also found one significant association between a variable and *lowered* risk of COVID-19. The variable was *atopic disease* (i.e., allergy conditions such as eczema/dermatitis, hay fever/allergic rhinitis, and asthma). "The only longstanding conditions associated with disease risk in our study," the researchers reported, "were atopic diseases, which were associated with reduced risk of disease, particularly among those who also had asthma." They speculated that this result might be explained by decreased expression of ACE2—the receptor SARS-CoV-2 binds to in infection—a condition reported in people with high allergy sensitivity and asthma.[370]

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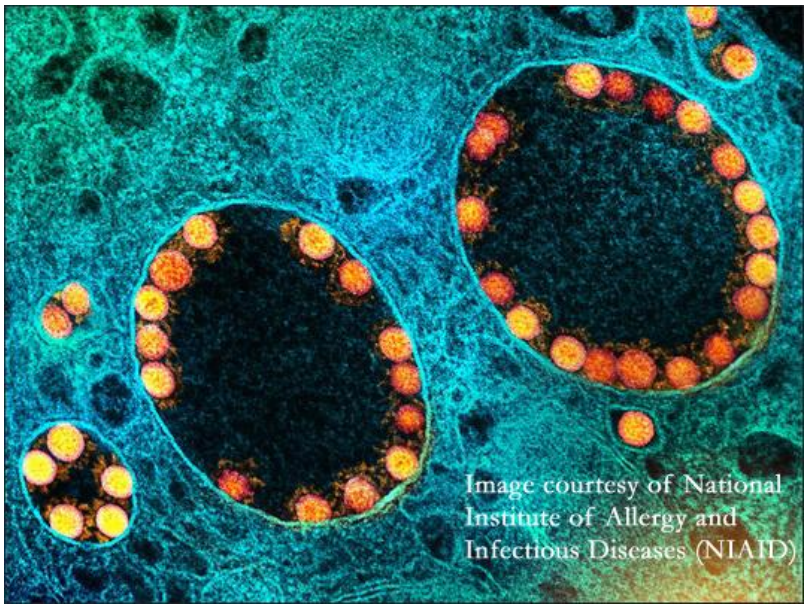
[369] Elliott et al., "COVID-19 Mortality in the UK Biobank Cohort."

[370] Holt et al., "Risk Factors for Developing COVID-19." Quote is from p. 12 (also see p. 5). The adjusted odds ratio (aOR) for atopic disease was 0.75 and for asthma 0.62.

One of the astounding realities of viral infection is just how much damage so little viral material can prompt. It has been estimated that the total mass of SARS-CoV-2 in a body during peak infection is still substantially less than the mass of a single poppy seed.[371] Yet this incredibly tiny amount of virus can wreak all kinds of havoc on the human body. Let's start simply. COVID-19 remains most widely known as a respiratory illness with symptoms like those associated with pneumonia. These include:

- cough (including coughing up blood);
- fatigue;
- fever;
- headache;
- myalgia (muscle pain);
- phlegm coughed up from the lungs (sputum production); and,
- shortness of breath.

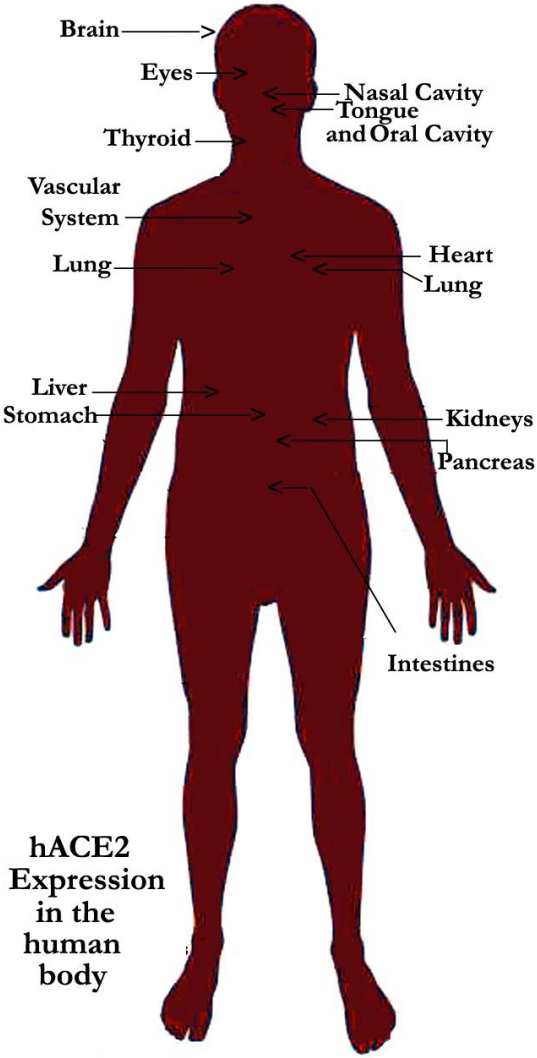
Coronaviruses easily find host cells along the human airway epithelium (HAE)—the cells lining the respiratory tract. The above list reflects the invasion of epithelial cells in the respiratory tract, which then leads to virus replication and migration downward to the airways, where it enters alveolar epithelial cells in the lungs. Perhaps another image will make the situation more vividly real. Below is a transmission electron micrograph of COVID virus particles (in gold) within endosomes of a heavily infected nasal Olfactory Epithelial Cell.



[371] Sender et al., "The Total Number and Mass of SARS-CoV-2 Virions," 5.

The virus' invasion of an ill-prepared immune system can lead to such a strong (one might metaphorically say 'panicked') response that a condition known as 'cytokine storm syndrome' occurs—a life-threatening inflammatory response that kills tissues the immune system ordinarily protects. The result is the severe acute respiratory syndrome (SARS) that is the first part of the virus' name.[372]

However, it quickly became apparent that COVID-19 also is associated with a disturbingly wide-range of other symptoms, across a range of organs, because SARS-CoV-2 binds to hACE2 receptors found throughout the body:



[372] Hu et al., "Characteristics of SARS-CoV-2 and COVID-19," 147.

Coordinating the body locations with symptoms produces a list that includes all of the following as documented occurring with COVID-19.[373]

Organs affected	*Symptoms (arranged alphabetically)*
Brain	brain inflammation, confusion, seizures, and strokes
Ears	hearing loss, tinnitus, vertigo
Eyes	conjunctivitis, inflammation of the membrane that lines the front of the eye, and inner eyelid
Heart and blood vessels	arrhythmias, blood clots, cardiac inflammation and injury, chest distress/pain, elevations of cardiac injury biomarkers, heart failure, lymphopenia (an abnormally low number of lymphocytes (a type of white blood cell)), and palpitations (*palmus*)
Intestine and stomach	abdominal pain, anorexia, diarrhea, nausea, and vomiting
Kidney	abnormal creatinine level, protein and blood in urine
Liver	abnormal enzymes levels
Nose	partial or full loss of smell (*anosmia*)
Pancreas	pancreatitis
Skin	chilblain-like lesions
Toes/Feet	rash as red or purple lesions on side or sole of foot

SARS-CoV-2 wreaks so much havoc because it has so many places it can be—and so many of these places are vital to human health and well-being.

Distinctive Symptoms

In the early variants as with the original Wild Type virus, the most distinctive symptoms were those tied to its name—severe, acute, *respiratory* syndrome. The lungs seemed to be 'COVID central' and images of people on mechanical ventilators were common in media accounts.

Then, as noted above, as time when on and the pandemic spread and persisted, more and more symptoms were established as features of the disease.

But COVID-19 became especially associated with two other distinctive symptoms—ones that at first blush might seem unexpected, such as a *loss of smell* (olfactory dysfunction, or OD) and/or *loss of taste* (both elaborated later). A study in the Spring of 2020 found prevalence (commonness) of a mild form of OD to be 85.9%, and disappeared within six months in 95% of patients.[374]

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[373] Both the pneumonia associated symptoms and symptoms of other organs are adapted from Hu et al., "Characteristics of SARS-CoV-2 and COVID-19," 148, and Salamanna et al., "Body Localization of ACE-2," 1. On ear symptoms, see Trafton, "Study." On skin lesions see Yorulmaz and Aktas. "SARS-CoV-2-induced Chilblains." On so-called 'COVID-toe' see Karlis, "What Is 'Covid Toe'?" and Mazzotta, Trocolli, and Bonifazi, "Idiopathic Chilblains and COVID Toes."
[374] Leichen et al., "Prevalence and 6-Month Recovery of Olfactory Dysfunction."

As the Delta variant became displaced and the Omicron variant gained ascendancy it became apparent that COVID-19 was taking on a distinctively new profile. Instead of making the lungs 'COVID central' the new variant had moved higher and established its apparent base of operations in the *upper airways*.

At the end of 2021, a symptom supposedly distinctive to it was reported. *Night sweats* were reported in an early study of 212 Chinese COVID-19 patients, which found 114 (53.8%) experienced 'profuse sweating'—and of those 102 (89.5% of the sweating group; 48% of the entire group) experienced night sweats.[375] This sweating, so severe a person's clothing is soaked through, was reported by doctors in both South Africa and the United Kingdom. Night sweats were reported as one of the most common symptoms due to Omicron. Still, many other common symptoms associated with Omicron—fatigue, muscle aches, and sore throats—are of a kind found with other variants.[376]

At the beginning of 2022, an analysis of reports from December, 2021, of positive cases from the United Kingdom using the ZOE COVID Study app reported the following as the top five symptoms associated with Omicron:

- runny nose;
- headache;
- fatigue (either mild or severe);
- sneezing; and,
- sore throat.

That analysis also found such classically distinctive symptoms such as loss of smell and/or taste to be infrequent with Omicron.[377]

In the United States, early data from 37 symptomatic patients infected by the Omicron variant showed the following (ranked by frequency):

Symptom	Number of Instances	Percentage (%)
Cough	33	89%
Fatigue	24	65%
Congestion or runny nose	22	59%
Fever	14	38%
Nausea or vomiting	8	22%
Shortness of breath or difficulty breathing	6	16%
Diarrhea	4	11%
Loss of taste or smell	3	8%

Only one of the 37 patients was hospitalized.[378]

[375] Zhang et al., "Gastrointestinal Tract Symptoms in Coronavirus Disease 2019."
[376] Scribner, "Doctor Reveals New Nightly Omicron Variant Symptom."
[377] ZOE editorial staff, "What Are the Symptoms of Omicron?"

Additional Notes on Various Symptoms

The range of COVID-19 symptoms and body areas involved make it impractical to elaborate here on all of them. However, some can be briefly noted here.

Central Nervous System (CNS)

Early on one of the puzzles of COVID-19 for many people was why a *respiratory* disease would result in so many *neurologic* symptoms. As explained in the previous chapter, SARS-CoV-2 attaches at hACE2 receptor sites, which are found in many places in the body. But we also found that cofactors facilitate the viral processes of attachment and entry. One, we saw, is neuropilin-1, which facilitates entry of SARS-CoV-2 into the central nervous system (CNS) and is highly expressed in some areas of the brain (e.g., hippocampal formation).[379] On the other hand, by early 2022 most of the evidence suggested it isn't viral transmission as such that causes the problems, but immune system activation and resulting inflammation within the CNS.[380] Nevertheless, also in early 2022, evidence from brain scans comparing changes from pre-COVID-19 to post-COVID-19 indicated changes in brain structure that might implicate the virus.[381]

Among CNS symptoms associated with COVID-19 are these:

agitation	encephalopathy	ischemic strokes
confusion	hemorrhage	seizures
edema (fluid buildup)	impaired consciousness	vision disturbances

In fact, a retroactive case series study of 214 hospitalized patients with confirmed cases of severe COVID-19, 45.5% of them experienced impaired consciousness and other neurologic symptoms.[382]

Neurologic manifestations have been observed in 42% of COVID-19 patients at disease onset—and increased incidence is associated with hospitalization (63%). Altogether, some 82% show some neurologic manifestation in the course of their illness. Often these manifestations are mild (e.g., dizziness, headache, diminished sense of taste (*hypogeusia*), diminished sense of small (*hyposmia*), or nerve pain (*neuralgia*)), but they can be severe (e.g., encephalitis or stroke).[383]

CNS manifestations can lead to complications. Near the end of 2021, at the annual meeting of the Radiological Society of North America, researchers reported that an estimated 1% of hospitalized COVID-19 patients will probably show *nervous system complications*. Magnetic Resonance Imaging (MRI) and Computed Tomography (CT) studies found CNS abnormalities associated with COVID-19 in 1.2% of patients studied. The most common abnormality was stroke

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[378] CDC, "SARS-CoV-2 B.1.1.529 (Omicron) Variant," 1733 (see Table 3 for data used here). .
[379] Davies et al., "Neuropilin-1 as a New Potential SARS-CoV-2 Infection Mediator," 4223–25.
[380] Spudich and Nath, "Nervous System Consequences of COVID-19," 268.
[381] Douaud et al., "SARS-CoV-2 Is Associated with Changes in Brain Structure."
[382] Davies et al., "Neuropilin-1 as a New Potential SARS-CoV-2 Infection Mediator," 4222.
[383] Whitmore and Kim, "Understanding the Role of Blood Vessels," 1947.

from clogged arteries, but bleeding in the brain, inflammation, and other issues were sometimes seen.[384]

Not long after vaccines were approved in the U.S. there were scattered reports of blood clots and strokes as adverse events. With respect to CNS blood clots (*central nervous system thrombosis* (CVST)), of 54 million recipients of the Pfizer vaccine there were 35 *possible* cases (about 0.00006%); of the Moderna vaccine there were 5 possible cases out of 4 million recipients (0.000125%).[385] Nevertheless, anti-vaxxers seized on the remote possibility of such an adverse event to argue against vaccination as being too risky. What anti-vaxxers didn't point out is that the risk of such events is *higher among the unvaccinated* who contract COVID-19. Epidemiologist Julia Hippisley-Cox observes that U.S. research found the risk of CVST associated with COVID-19 infection was about *seven times greater* than that associated with either the Pfizer of Moderna vaccines.[386] After reviewing the research Richard Francis, Head of Research at the Stroke Institute, remarked that the benefits of vaccination for *reducing* stroke risk from COVID-19 is so far greater as to justify vaccination on its own.[387]

Eyes and Ears

The SARS-CoV epidemic earlier in the century demonstrated that coronavirus particles could be found in the tears of the infected. It was not a great leap, then, to suspect SARS-CoV-2 infection might manifest in the eyes. In fact, an *ex vivo* study (lit., 'outside the body'—but with attention to minimizing alteration of natural conditions) found the highest viral replication 48 hours after infection was in conjunctival tissue. At the beginning of the pandemic one researcher estimated an *ocular manifestation* (e.g., excessive watering of the eye [*epiphora*], enlargement of conjunctival vessels [*conjunctival injection*], and eye irritation [*chemosis*]) might occur in nearly a third (30%) of COVID-19. Subsequent analysis in a systematic review and meta-analysis revised this estimate sharply downward, and found no association between this kind of symptom and severe COVID-19. It is worth noting that the eye can provide an entry point for SARS-CoV-2 because hACE2 receptors are found in conjunctiva (the membrane lining the inside of the eyelids and covering the front of the eye), and cornea (the protective outer area of the eye).[388]

The ears connection with COVID-19 is an uncertain one as no underlying physiology is readily seen for symptoms that have been reported by patients. Tinnitus (ringing in the ears) and equilibrium disorders such as dizziness and vertigo have been reported. An Italian study involving 185 patients within 1–2 months of COVID-19 diagnosis found more than a fifth (23.2%) reported tinnitus. A smaller number of patients (18.4%, or a little more than 1-in-6) re-

[384] Lapid, "Brain Problems Found in 1%."
[385] Hippisley-Cox, "Risk," 10.
[386] Hippisley-Cox, "Risk," 2.
[387] Science Media Centre. "Expert Reaction to Study."
[388] La Distia Nora et al., "Are Eyes the Windows to COVID-19?"

ported an equilibrium disorder and in that group the disorder was usually dizziness (94%), though a few reported acute attacks of vertigo (5.9%). A portion (7.6%) of patients experienced both tinnitus and an equilibrium disorder.[389] Because tinnitus is exacerbated by stress it may be that its experience alongside COVID-19 can be explained by that fact.[390] On the other hand, a study of 53 people—26 COVID-19 positive and 27 in a control group—none with previously reported audio-vestibular issues, found in some aspects of this system significant differences between the COVID-19 positive patients and those in the control group. The researchers suggest this might be explained by neural tissue invasion by the virus or by inflammation of the blood vessels (*vasculitis*).[391] It is interesting to note that the possibility of a COVID-related skin rash of the ear also has been reported. This may be an example of a still mysterious phenomenon—dermatologic manifestations associated with COVID-19.[392]

Gastrointestinal System

One study presenting a systematic review and meta-analysis of 26 studies focused on the prevalence of nausea, vomiting, diarrhea, abdominal pain, and anorexia. It reported the following (listed from most to least prevalent*):

Condition	Prevalence
anorexia	17%
diarrhea	8%
nausea and vomiting	7%
abdominal pain	3%[393]

*'Prevalence' refers to the total number of people in a group who have the condition or show the symptom at the time of measurement (or during some specified period of time).

Other researchers who conducted a different systematic review and meta-analysis—this one of 35 studies, encompassing 6,686 COVID-19 patients—found that perhaps 10% present *only* gastrointestinal symptoms, without respiratory symptoms—a situation that may delay diagnosis. Both *digestive symptoms* and *liver injury* are not uncommon: some 15% of patients reported gastrointestinal symptoms, with nausea or vomiting, diarrhea, and loss of appetite the most common. Liver injury was found in 19% of patients. Moreover, some research indicates that patients with digestive symptoms might experience worse outcomes than those without. Such symptoms were also correlated with higher rates of complications of acute respiratory distress syndrome and liver injury.[394]

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[389] Viola et al., "Tinnitus and Equilibrium Disorders in COVID-19 Patients."
[390] Chirakkal et al, "COVID-19 and Tinnitus," 1614.
[391] Tan et al., "Effects of COVID-19 on the Audio-Vestibular System."
[392] Phamduy, Young, and Ramolia, "Localized Scarlatiniform Rash of the Ears."
[393] Dong et al., "The Prevalence of Gastrointestinal Symptoms," 68, and see 73.
[394] Mao et al., "Manifestations and Prognosis of Gastrointestinal and Liver Involvement."

More importantly, perhaps, should be a recognition of *inter-organ crosstalk*. This idea emphasizes the role in disease progression of links between parts of the body that are traditionally considered separately. For example, COVID-19 has been viewed primarily as a respiratory disease and so focus has been upon the lungs. But some researchers have suggested looking at a 'gut-lung axis.' They point out that both are *mucosal sites*—body places where mucus membranes have surfaces composed of epithelial cells, where hACE2 receptors abound. Cross-mucosal interactions between respiratory mucosa and intestinal mucosa are well-documented. This 'cross-talk' in the body suggests not only why gastrointestinal symptoms occur with COVID-19, but also that each part may influence the other in such matters as an inflammatory response to infection.[395]

Heart and Blood Vessels

Cardiac dysfunction has been observed in up to a quarter (25%) of COVID-19 patients. One research study noted that most hospitalized COVID-19 patients show abnormal echocardiograms—and even after recovery a majority, as evidenced by MRI, still suffer from impaired cardiac function. This study reported that the virus disrupts the structure of heart muscle tissue (*myofibrillar structure*) and its ability to contract normally (*cardiac contractile machinery*).[396] Another study found that rather than the main coronary arteries, SARS-CoV-2 targets the small blood vessels (e.g., epicardial capillaries) of the cardiovascular system, where hACE2 expression is high. In such places COVID-19 induced immune-system produced inflammation (*endotheliitis*), suggesting that such systemic inflammation may be the major cause of some of the cardiac complications (e.g., heart arrhythmias and myocardial injury) seen in the disease.[397]

Many observations have been made with respect to COVID-19 and the body's blood transportation system. For example, a study involving more than 10,000 hospitalized patients found that testing positive for COVID-19 correlated with a 3.88-fold increased odds of developing a laboratory-confirmed bloodstream infection (LCBH).[398]

Earlier we looked at blood clots in the CNS leading to stroke. But blood clots occur in various tissues and organs of COVID-19 patients. Not surprisingly, this occurrence is linked to high expression of hACE2 found in the membrane lining the inside of blood vessels (the *endothelium*). SARS-CoV-2 infection leads to injury inside vascular walls of blood vessels, which in turn is associated with blood clotting (*coagulation*).[399] Abnormal blood clotting in a condition known as *disseminated intravascular coagulation* (DIC)—something documented as common in deceased COVID-19 patients.[400]

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[395] Zhang et al., "COVID-19: Gastrointestinal Symptoms from the View of Gut-Lung Axis."
[396] Perez-Bermejo et al., "SARS-CoV-2 Infection," 1, 8.
[397] Maccio et al., "SARS-CoV-2 Leads to a Small Vessel Endotheliitis in the Heart," 1, 7–8.
[398] Shukla et al., "Bloodstream Infection Risk," 2593.
[399] Biswas et al., "Blood Clots in COVID-19 Patients."
[400] Ponti et al., "Biomarkers Associated with COVID-19 Disease Progression," 391.

We saw earlier that heart specialists Peter Libby and Thomas Lüscher expressed their estimation of COVID-19 in a 2020 journal article: "COVID-19 is, in the end, an endothelial disease."[401] In a healthy environment—one absent viral infection, for example—endothelial cells are able to prevent coagulation and inhibit damaging inflammation. COVID-19 involves tissue damage from viral infection that induces microvascular inflammation. Infected endothelial cells undergo *endothelial activation*—a process of interaction with the immune system that leads to inflammation and loss of vascular integrity producing disrupted cell membranes and serious cell injury.[402]

Kidneys and Liver

The *kidneys*, important to cleansing the body and preserving its balance of fluids, salts and minerals, are not uncommonly impacted by COVID-19. Research suggests most often the suffering of the kidneys from the disease is evident by elevated levels of protein in the urine (*proteinuria*) or blood in the urine (*haematuria*), both indicating *acute kidney injury* (AKI). Such injury has been observed in 20–40% of COVID-19 patients in ICU admissions in Europe and the U.S. This means it is considered an indicator of severe COVID-19.[403]

A systematic review and meta-analysis of 14 studies found in many COVID-19 patients *abnormal liver function*—measured by tests (aspartate transaminase (AST); alinane transaminase (ALT); and bilirubin (TBil)). Increased levels of AST were found in 24%, of ALT in 25%, and of TBil in 13%.[404]

Skin

Skin manifestations can be classified into groups (from most to least severe):

- lesions caused by blood vessel blockage (*vaso-occlusive*)—least common, but associated with severe disease (survival rate of 78.9%);
- bubble-like sacs under the skin, such as cause red spots (*vesicular lesions*)—most often at COVID-19 onset (survival rate of 96.1%);
- rash caused by injured or inflamed blood capillaries (*erythematous rash*)—mostly on trunk of body (survival rate of 98.2%);
- hives (*urticarial lesions*)—transient (less than 24 hrs.), mostly in middle-aged women (survival rate of 97.5%); and,
- itchy, red patches like chilblains (*pseudo-chilblains*)—so-called 'COVID-toes' the best known example (survival rate of 98.7%).

Frequency of skin manifestations varies from least common in people of Asian descent (3.1%) to most common in those of European descent (96.9%).[405]

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[401] Libby and Lüscher. "COVID-19 Is, in the End, an Endothelial Disease."
[402] Whitmore and Kim, "Understanding the Role of Blood Vessels," 1949; also Hunt and Jurd, "Endothelial Cell Activation."
[403] Ahmadian et al., "Covid-19 and Kidney Injury."
[404] Dong et al., "The Prevalence of Gastrointestinal Symptoms," 68, and see 74.
[405] Tan, Tam, and Oh, "Skin Manifestations of COVID-19." Cf. Vásquez-Osorio et al., "Pseudo-Chilblain Lesions and COVID-19."

Finally, we can conclude this examination of symptoms by looking at a pair that have become well-noted as distinctive features of COVID-19: loss of smell and loss of taste.

One of the distinctive features of COVID-19 is a loss of smell, formally termed acute *olfactory dysfunction* (OD), but also sometimes referred to as *anosmia* (literally, 'without' (*an-*) 'smell' (*-osmē*)). In May, 2020, anosmia was formally recognized as a symptom of COVID-19 based on an accumulation of evidence that suggested a loss of smell occurred in more than half (55%) of COVID-19 patients. This loss was typically sudden and complete, but also transient, passing with recovery. However, about 1-in-10 (10.6%) reported this effect lingering more than a month.[406]

In 2021, a meta-analysis of its incidence (i.e., likelihood of occurrence in a period of time) in COVID-19 patients found it to be 52.7%—a bit more than half of them. Of this group most recover their sense of smell in time, but as noted above some develop *chronic olfactory dysfunction* (COD), meaning a persistence of the condition over a more extended time than expected (e.g., 6 months or longer). Analysis of COVID-19 data for the United States between January 13, 2020 and March 7, 2021, led to an estimate of between 700,000 up to 1.6 million Americans experiencing COD—and the authors of the study note the true number of cases may be far higher.[407]

Loss of taste is known by the medical term *ageusia* (literally, 'loss' (*a-*) of 'taste' (*geusis*)). A systematic review published in 2021, which reduced an initial pool of 298 articles to 17 which met the study's criteria, found that while substantial variation was reported in when this occurred, many patients who experienced loss of taste and/or smell did so 4–5 days after the onset of other symptoms. There also was substantial variation in how long symptoms persisted, though an average of 1–2 weeks was common. Variability among the studies examined made it difficult to know how often these losses occur together.[408]

Because something like loss of smell and/or can also occur for other reasons, this one symptom should not be assumed due to COVID-19. Diagnostic tests can help establish the cause and guide treatment.[409] But this symptom—as well as that of loss of taste—may have some predictive value. An analysis of 2,618,862 reports by people using a smartphone-based application to report possible COVID-19 symptoms found that loss of small and taste was far more likely to be reported by those who tested positive for COVID-19 (65.03%) than for those who did not (21.71%).[410]

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[406] Walker et al., "Ansmia and Loss of Smell in the Era of COVID-19," 1.
[407] Kahn, Kallogjeri, and Piccirillo, "Growing Public Health Concern."
[408] Assunção Santos et al., "Onset and Duration of Symptoms of Loss of Smell/Taste."
[409] Walker et al., "Ansmia and Loss of Smell in the Era of COVID-19," 1–3.
[410] Menni et al., "Real-Time Tracking of Self-Reported Symptoms."

Course of Infection

General Remarks

As an enemy SARS-CoV-2 is especially worrisome because of its high success in evading detection while rapidly spreading in a host body. But added to that reality is that it is highly transmissible from one person to another because the viral load in the host is so high even before the person feels sick.

Incubation and Infection Period

The incubation period for COVID-19 is variable. Some cases are asymptomatic, but when symptoms occur they might be anywhere from 2–14 days after infection.[411] An early study reported a median incubation period of 5.1 days, with 97.5% of symptomatic cases showing sign of infection within 11.5 days.[412] Incubation periods seem to vary slightly from variant to variant, with the incubation period a little shorter for Delta than for earlier variants Alpha, Beta, and Gamma.[413] Early indications were the period was even shorter for Omicron.[414]

The 'serial interval' (i.e., the time between symptom onset in the first infected person and the symptom onset in the next infected person) median is about four days. That is actually a shorter time than the estimated incubation period, which means *transmission of the virus to others is likely before the first person experiences any symptoms*.[415] In fact, some research has indicated it might be as high as 79% of documented cases that stem from undocumented infectious persons in the asymptomatic or mild symptom periods.[416]

COVID-19 can spread before there is any obvious evidence it even is present. In technical terms, SARS-CoV-2 infectiousness is highest before seroconversion—the presence of antibodies in the blood—occurs. Worse, it may even be the case that the contagious period for COVID-19 persists for as much as a week *after* recovery from symptoms.[417]

The virus is shed through particles sent out into the air (by speaking, singing, coughing, etc.) and unsuspectingly taken up into the respiratory tract of another person. There the virus readily finds the hACE2 receptor sites it needs to begin a new invasion. The odds of becoming infected are affected by a variety of factors, such as how many infected people one is exposed to, the proximity of exposure, the duration of exposure, and so on. Nevertheless, within a very short period of time—by mid-February of 2020—the highly infectious nature of SARS-CoV-2 was recognized as a global pandemic.[418]

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[411] McClymont and Hu, "Weather Variability and COVID-19 Transmission," 2.
[412] Lauer et al., "The Incubation Period of Coronavirus Disease 2019 (COVID-19)."
[413] Grant et al., "Impact of SARS-CoV-2 Delta Variant on Incubation," 5, 9.
[414] Discovery Health, "Discovery Health."
[415] Nishiura, Linton, and Akhmetzhanov, "Serial Interval." Also see Du et al., "Serial Interval."
[416] Hu et al., "Characteristics of SARS-CoV-2 and COVID-19," 148.
[417] Ryding, "What Is Seroconversion?"
[418] Sanche et al., "The Novel Coronavirus."

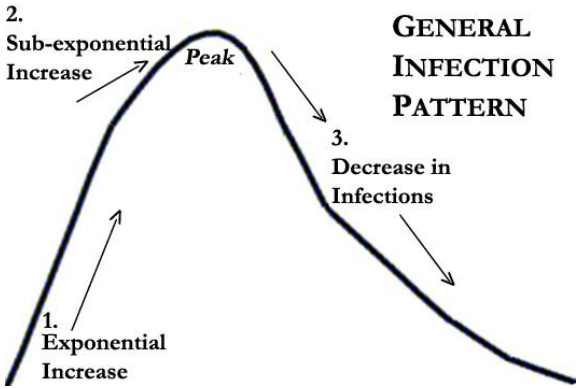
COVID-19's peak infection period is about a week (5–7 days). As noted in the preceding chapter, it has been estimated that a person afflicted with COVID-19 carries between a *billion* (10^9) and a *hundred billion* (10^{11}) virions during peak infection.[419] After infection antibodies circulating in the blood can be detected by certain tests (discussed in the chapter on the immune system). These show antibody levels peak within 3–5 weeks after infection, then begin to wane at variable rates among individuals.[420]

The course of infection in COVID-19 is highly variable.

With respect to the duration of infectiousness, CDC guidelines as of Fall, 2021, state: "For most children and adults with symptomatic SARS-CoV-2, the virus that causes COVID-19, infection, isolation, and precautions can be discontinued 10 days after symptom onset and after resolution of fever for at least 24 hours and improvement of other symptoms."[421]

General Infection Pattern

In terms of spread within a given geographical area, or country, everywhere COVID-19 has produced a significant number of infections it seems to have adhered to a discernible pattern, influenced not only by biology but by social behavior. The pattern seems to have two broad phases, first of rapidly increasing infections, followed by a slowing in growth as public measures like social distancing occur, and eventual decrease to a point where the number of daily infections are less than the number of daily recoveries.[422] It might be plotted graphically in three steps around a 'peak' where daily or weekly numbers of infection reach their height. This pattern resurfaces in each successive pandemic wave.



This picture of broad societal pattern needs to be accompanied by the far more varied pattern found in individual infection. The following illustration shows infection through a serious of disease stage progressions.

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[419] Sender et al., "The Total Number and Mass of SARS-CoV-2 Virions," 2.
[420] CDC, "Science Brief: SARS-CoV-2 Infection-Induced and Vaccine-Induced Immunity."
[421] CDC, "Ending Isolation."
[422] Miotto et al., "Does Blood Type Affect the COVID-19 Pattern?" 2.

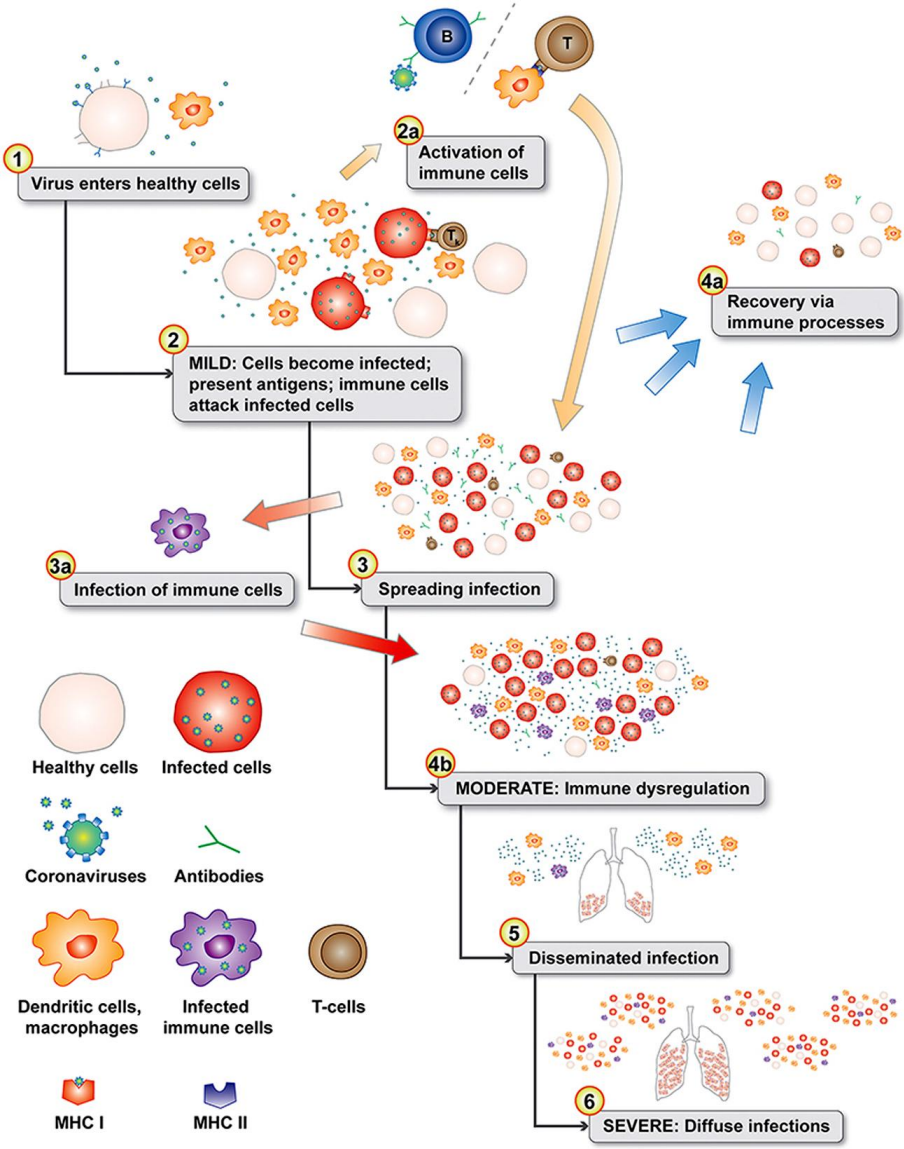

FIGURE 2 | Disease progression model with normal immune responses during the initial mild symptoms phase (see 1–3). Antigen presenting cells migrate to the lymph nodes to activate T cells (2a). The progression gate to moderate and server disease is the infection of phagocytic immune cells (3a) leading to immune dysregulation (4b). In the lungs, chemokines attract additional dendritic cells and immature macrophages that are subsequently infected in an positive feedback-loop infection cascade (4b). Infected phagocytic immune cells disseminate throughout the body infecting additional organs (5 & 6). Levels of chemokine and cytokines in the lungs from infected cells can create a cytokine storm (6). (Illustration from Darrell O. Ricke, "Two Different Antibody-Defense Enhancement (ADE) Risks for SARS-CoV-2 Antibodies." *Frontiers in Immunology* 12 (Feb., 2021) art. 640093. Made available through Creative Commons Attribution (CC-BY) license (https://creativecommons.org/licenses/by/4.0). No changes have been made to the figure.)

Disease Progression

Even nearly two years into the COVID-19 pandemic there is much yet to be learned about the disease. Let's start with a little biology to help make sense of the symptoms of COVID-19 that emerge. The main course of action by SARS-CoV-2 has been to bind to respiratory tract epithelial cells—those surface cells lining body tissue important to body defense against foreign material. The respiratory tract is the passageway by which we breathe and as the virus replicates it migrates down that path to the alveolar epithelial cells (AEC) of the lungs where it can trigger a strong immune response.[423] These AEC are of two types (Type I and Type II) and it has been hypothesized that SARS-CoV-2 especially targets AEC Type II. Although only 10–15% of AECs these Type II are important in stabilizing the airway epithelial barrier, airway regeneration during lung damage, and immune defense. As part of that defense they produce surfactant proteins (SPs) important for lung function. The damage done to these cells can lead ultimately to Acute Respiratory Distress Syndrome (ARDS).[424] This kind of picture helps explain the biology underlying what patients experience—sore throat, cough, difficulty breathing, and so forth. With the advent of the Omicron variant this typical picture was modified somewhat. Omicron appears to favor staying in the upper respiratory tract rather than concentrating in the lungs.

One innovative research study used internet search patterns to examine information from 32 countries across 6 continents with respect to COVID-19 disease progression. That analysis presents a picture like this:

Symptom Onset	→	**Symptom Worsening**
(fever, dry cough, sore throat, chills)	**5.22 days** (avg. days)	(shortness of breath)

They found such search patterns produce a picture mirroring that reported in the scientific literature, where labored breathing (*dyspnea*) typically occurs about 5 days after symptom onset.[425] Of course, typically clinical observation of symptoms as well as various biomarkers is used to distinguish degrees of illness; but what people show concern about in their internet searches seems to mirror what clinicians see. Using both medical tests and observations, those infected with SARS-CoV-2 can be classified as members of one or another group labeled according to the severity of their illness. This is useful for tracking the disease and its effects at a large-scale level, and for guiding treatment at the individual level. We'll examine that more closely a bit later.

The above box presents a common pattern in mild and moderate cases. But sometimes symptoms worsen. Here we are aiming simply at a broad picture of disease progression using a 'worst case' scenario—that is, imagining a patient

[423] Hu et al., "Characteristics of SARS-CoV-2 and COVID-19," 147.
[424] Carcaterra and Caruso, "Alveolar Epithelial Cell Type II as Main Target." See esp. p. 2.
[425] Lu and Reis, "Internet Search Patterns Reveal Clinical Course of COVID-19."

who deteriorates from a mild case through a moderate one to a severe situation that turns critical.

Hospitalization

The actual percentage of patients with COVID-19 may not be extraordinarily high—the European Surveillance System (TESSY), for example, recorded only 7.5% of diagnosed cases between August, 2020–May, 2021[426]—but the burden on health care systems can be. Even as the nightly national news across 2020–2021 reported record numbers of hospitalizations severely taxing the national health care system, solid statistical evidence made plain that news media was not exaggerating the crisis. For example, research using data from more than 800 hospitals in the Premier Healthcare Database Special COVID-19 Release (PHD-SR) for the period March, 2020–March, 2021, found that of 4,899,447 hospitalized adults (i.e., age 18 or older), 11% (540,667) had a diagnosis of COVID-19.[427] But that additional influx of patients threatened to overwhelm a system not prepared to handle normal needs *plus* a pandemic. Admission to hospital because of COVID-19 often—but not always—signals a severe case. Many times moderate cases are admitted for one reason or another.

One study looked at 176 COVID-19 hospital patients who were all, broadly speaking, with 'severe' disease. But they could be differentiated into two subgroups ('severe' and 'critically ill'). The critically ill had the most severe disease progression. The distinction looked like this:

	Severe COVID-19	**Critically-Ill COVID-19**
Length of Hospital Stay	5–19 days (Median: 10)	6–12 days (Median: 8)
Mortality Rate	4.5%	78.2%
Inflammation Markers	lower	higher
Lung Function	higher	lower

A critical predictor in disease severity was *serum ACE2 activity*—that is, blood serum showing increased levels of this receptor's activity predicts severity.[428] This event was dramatically evidenced in one critically-ill patient reported in a case study, whose ACE2 activity was about 40 times higher than normal.[429]

The most notable characteristic of these patients was *the presence of a pre-existing underlying condition*. The study using PHD-SR data reported above found that 94.9% of patients hospitalized with COVID-19 had at least one underlying medical condition, with five being the most common:

[426] European Centre for Disease Prevention and Control (ECDC), "Clinical Characteristics of COVID-19)" (Sept. 7, 2021 update).
[427] Kompaniyets et al., "Underlying Medical Conditions," 1, 3.
[428] Fagyas et al., "Circulating ACE2 Activity Predicts Mortality and Disease Severity."
[429] Nagy Jr. et al. "A Dramatic Rise in Serum ACE2 Activity."

Underlying Medical Condition	Percentage of Patients
essential hypertension	(50.4%)
disorders of lipid metabolism	(49.4%)
obesity	(33%)
diabetes with complication	(31.8%)
coronary atherosclerosis and other heart disease	(24.9%)[430]

(This list is comparable to other findings. For example, research in 2020 of 245 patients hospitalized before January 31, 2020, in two Chinese hospitals, found hypertension the most common comorbidity, followed by diabetes and coronary heart disease.[431])

Some of these five conditions are less familiar than others to most people. *Essential hypertension* (or, *primary hypertension*) is high blood pressure (>140/90) without a known secondary cause. It is associated with being overweight, stress, lack of exercise, and poor diet. *Disorders of lipid metabolism* are inherited, typically screened at birth, and include Gaucher disease and Tay-Sachs disease. *Obesity* is typically assessed as a body-mass index (BMI) of over 30 (see earlier discussion in this chapter). *Diabetes with complication* is exactly as the terms suggest: the primary disease of diabetes accompanied by one or another complication, such as heart disease, stroke, or kidney disease. *Coronary atherosclerosis* is one kind of heart disease, and it is wise to consider all heart disease as a serious underlying condition with respect to COVID-19. Atherosclerotic cardiovascular disease (ASCVD) results from high levels of so-called 'bad' cholesterol (LDL-C)—the kind that clogs arteries and leads to heart disease.

A predictable element in risk of hospitalization has been vaccination status. Those unvaccinated stand at much greater risk of hospitalization. For example, in the period of June–September, 2021, a time when the Delta variant was showing signs of decline but before Omicron burst onto the scene, a study reported 85% of those hospitalized with COVID-19 were unvaccinated.[432]

Death

COVID-19 kills. A latter section, on mortality, examines this reality further. Here the focus is somewhat different. Death is considered here as one end stage development from COVID-19 disease progression. Therefore our focus here is on matters related to disease progression and not such matters as individual risk for death or overall mortality statistics.

An early study of Chinese patients stricken with COVID-19 found that the median time from illness onset to death was 18.5 days, with a typical range of from 2–3 weeks.[433] Death from COVID-19 is commonly preceded by *intubation*

[430] Kompaniyets et al., "Underlying Medical Conditions," 3.
[431] Zhou et al., "Clinical Course and Risk Factors," 1057.
[432] Khullar, "How to Think about the Risks of Omicron."
[433] Zhou et al., "Clinical Course and Risk Factors," 1057.

and invasive mechanical ventilation (IMV)—a procedure by which a machine ventilator pumps air through a tube down the throat into the lungs to assist breathing. In 2020 it was reported that more than 70% of critically ill COVID-19 patients received intubation and IMV support.[434]

Regrettably, intubation and IMV is truly a course of last result, with the generally expected outcome death. For example, in the study of early cases of Chinese hospitalized COVID-19 patients, the median time from onset of illness to IMV was about 2 weeks (14.5 days); 31 of the 32 patients given IMV died (97%).[435] Unfortunately, even as 2021 drew to a close, intubation and IMV was still closely associated with death.

Risk factors for death among those severely ill with COVID-19 include:

- comorbidity;
- obesity;
- anxiety and fear-related disorders; and
- diabetes with complications.[436]

Comorbidity refers to more than one condition of illness existing 'alongside' (*co-*) another 'condition of illness' (*-morbidity*). The yearlong PHD-SR study referred to above reported that across states of the U.S., the percentage of adults with two or more underlying conditions ranges from more than one-third (38%) to just under two-thirds (64%). It found that the risk of death was often incrementally higher as the number of underlying medical conditions rose.[437]

The fact that *anxiety and fear-related disorders* emerged as a major risk factor may seem surprising—and it remains poorly understood. The authors of the above study speculate that a number of factors may be at play, including the possible effects of medications on the immune system or cardiovascular system, which along with the disorder itself could contribute to a reduced ability to fight infection, or because COVID-19 exacerbates the disorder. But the researchers also point out they cannot exclude the possibility that COVID-19 itself gives rise to the diagnosis of a disorder of this kind (i.e., the person did not meet the criteria for such a disorder prior to having COVID-19).[438]

Finally, it should be noted that SARS-CoV-2 can outlive its host. Cadavers from COVID-19 deaths can remain infectious long after death. One study found viral loads in the upper part of the throat (*nasopharyngeal region*) can remain stable for nearly three weeks (20 days) after death. Another found virus RNA up to 325 hours (13.5 days) postmortem—and sometimes at levels exceeding those measured in the acute phase of infection while the person was alive.[439]

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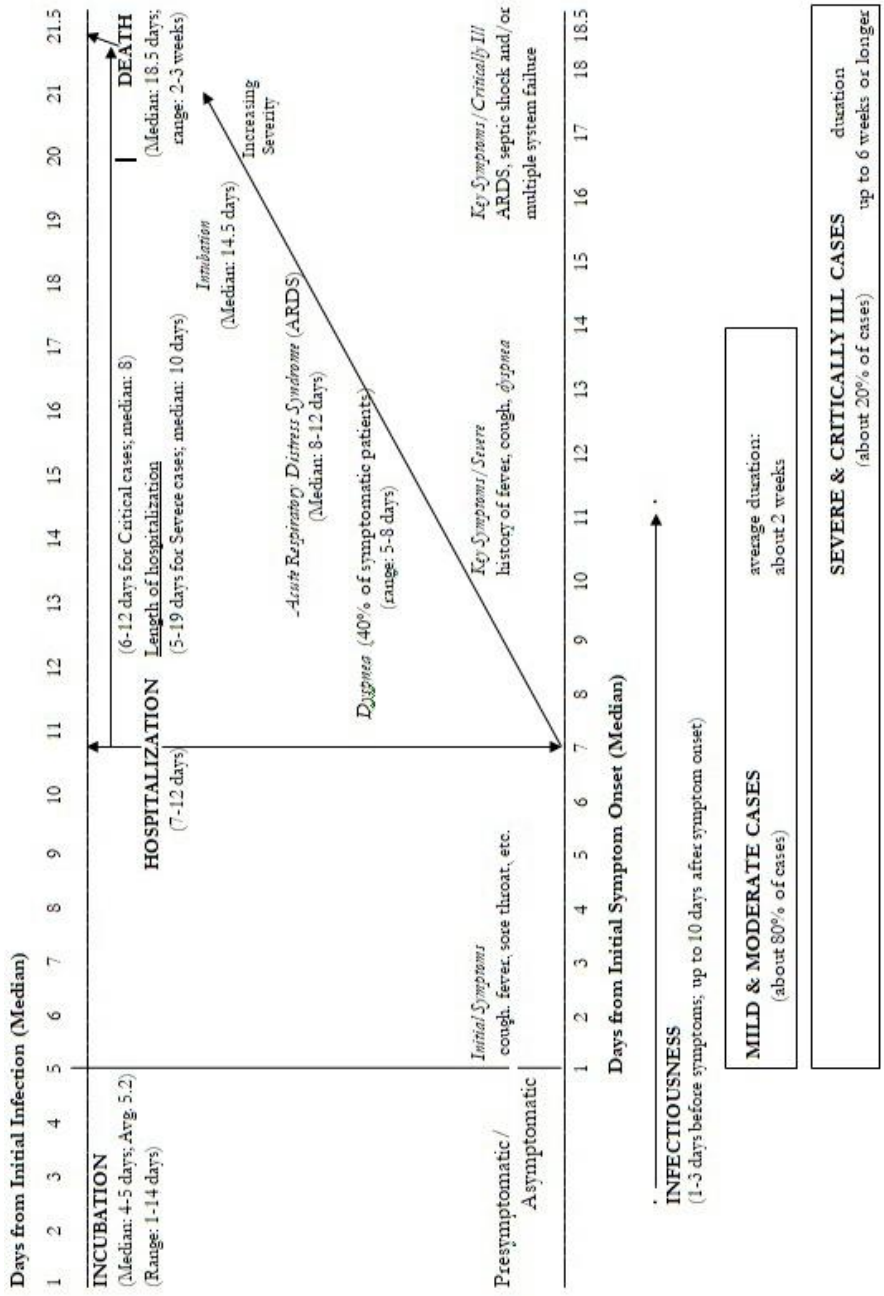
[434] Luo et al., "Intubation, Mortality, and Risk Factors," 1.
[435] Zhou et al., "Clinical Course and Risk Factors," 1057.
[436] Kompaniyets et al., "Underlying Medical Conditions," 1, 3, 5.
[437] Kompaniyets et al., "Underlying Medical Conditions," 5.
[438] Kompaniyets et al., "Underlying Medical Conditions," 5.
[439] Heinrich et al., "Postmortem Antigen-Detecting Rapid Diagnostic Tests to Predict Infectivity of SARS-CoV-2-Associated Deaths."

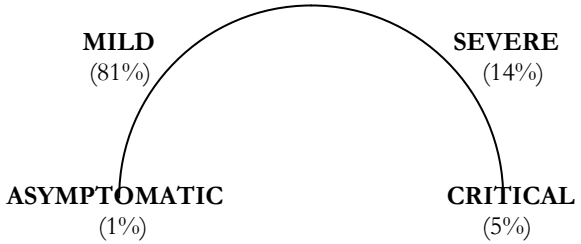
If we put all these matters together and add a timeline we can get a rough—*very* rough—picture of disease progression leading to death.[440]



[440] The numbers used here come from a number of studies but must be seen as *estimates* and general in character. The specific course of disease is highly variable by individual.

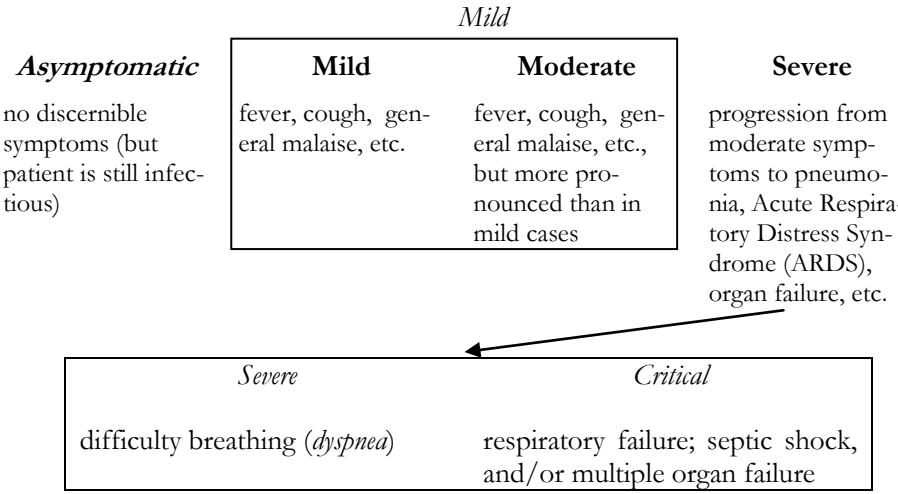
Classification of COVID-19 Cases

A study of Chinese patients reported early in the pandemic found the following distribution of four categories—asymptomatic, mild, severe, and critical:



'Mild' cases included both those without pneumonia *and* with mild pneumonia. 'Severe' cases included difficulty breathing (*dyspnea*) with blood oxygen saturation of 93% or less. 'Critical' cases involved respiratory failure, septic shock, and/or multiple organ failure.[441] The death rate in this group is the highest.[442]

COVID-19 cases are today often divided into four classes (in bold print): asymptomatic, mild, moderate, and severe. We can both broadly summarize them and correlate them with the above scheme (in italics) as follows.



Both schemes are best seen as a continuum (like pictured for the Chinese study), and not as sharply distinguished stages or classes with sharp borders.

Finally, some prefer to use five categories—asymptomatic, mild, moderate, severe, and critical (or critically-ill). This latter, more comprehensive scheme of

[441] Wu and McGoogan, "Characteristics of and Important Lessons," 1239.
[442] Gao, "COVID-19: Risk Factors for Critical Illness," 1, notes death rates in reports of 26%, 39%, 60.4%, and 85.43%. Also see Dan et al., "Risk Factors."

classification is the one presented on the National Institutes of Health (NIH) website. Their presentation at the beginning of 2022 can be pictured as follows:

Asymptomatic or Presymptomatic	Mild illness	Moderate illness	Severe illness	Critical illness
Positive test result absent COVID-19 symptoms	COVID-19 symptoms: cough diarrhea fever headache loss of smell loss of taste malaise muscle pain nausea sore throat vomiting *without* abnormal chest image; dyspnea on exertion; shortness of breath	[1] evidence of lower respiratory disease during clinical assessment or imaging; with [2] oxygen saturation (SpO_2) ≥ 94% on room air at sea level.	[1] oxygen saturation (SpO_2) ≤ 94% on room air at sea level [2] a ratio of arterial partial pressure of oxygen to fraction of inspired oxygen (PaO_2/FiO_2) <300 mm Hg [3] a respiratory rate >30 breaths/min., or lung infiltrates >50%	[1] respiratory failure (Acute Respiratory Distress Syndrome (ARDS)) [2] septic shock and/or [3] multiple organ dysfunction (e.g., cardiac dysfunction, exaggerated inflammatory response, and/or exacerbation of underlying comorbidities)[443]

Symptoms are listed alphabetically, not in terms of frequency of occurrence.

The Construction of a Classification System

Because the coronavirus was 'novel'—one not encountered previously—the response to it medically in terms of classifying disease presentation had to be accomplished 'on the fly.' Health systems had to rely on previous experience with other diseases while sculpting something appropriate to COVID-19. As we might expect, this led to some variation in how patients diagnosed with COVID-19 were classified, resulting not only in different classification schemes as shown above, but also making it possible that a particular patient classified one way in one place might be classified differently if diagnosed somewhere else. This is neither remarkable nor particularly worrisome. What matters is consis-

[443] Content from NIH, "Clinical Spectrum of SARS-CoV-2 Infection" (Oct. 19, 2021 update). Cf. Gandhi, Lynch, and Del Rio, "Mild or Moderate Covid-19," 1759 (Figure 1). **N.B**. Lie et al., "Risk Factors," 2, note the National Health Commission of China added a 4th criterion: patients with > 50.00% lesion progression in lung imaging within 48 hrs.

tency of diagnosis and classification within a particular system and enough generalizability that different systems can talk meaningfully with one another. In the end, while it is of interest to provide statistical estimations of how many sick people fall into one classification or another, it is far more important that within the health system where the person is classified the appropriate care is rendered.

It is beyond the scope of this volume to explore in any depth the different ways health systems and governments solved the problem of classification to guide treatment. However, it is worth a quick look at an example to get a sense of how this occurs. In the Republic of Korea (South Korea) the government used the health systems of the nation to gather information quickly and formulated a five factor system, where each variable was scored along a range from 0–3 points, yielding a possible total score from 0–15. Of the five factors, four were clearly and easily measurable (pulse rate as beats per minute; systolic blood pressure; respiratory rate as breaths per minute; and body temperature), while the fifth (level of consciousness) was assessed by clinical observation of responsiveness. The result led to a scoring system: scores of 0–4 were classified as 'mild'; 5–6 were 'moderate'; scores 7 or above were 'severe' or 'very severe.'[444]

As we will see, the classification labels and descriptions above combine both clinical observations, which are more subjective by nature, with test measurements, which are more objective. In other words, just like what South Korea did, health care systems take what is readily available, such as what doctors see in their patients, and combine them as well as they can with tests that probe what cannot be easily seen. Those tests, typically, require time for processing and interpretation. So as a practical course of action the following occurs:

- *An initial emphasis is put on confirming infection*. This means report of symptoms with a diagnostic test (e.g., antigen or molecular test).
- *After confirmation the most important matter is determining whether the case is serious enough to warrant hospitalization*. Thus the decisive line is that separating mild or moderate from severe or critical. Accordingly tests accompany clinical observation to measure essential functions like respiration.
- *Following decision as to whether the case is severe further discrimination can be made to classify a case more precisely*. This is done based on clinical observation and biomarkers (measured by various tests).
- *The key is remembering the primary purpose of diagnosis and classification, which is to guide treatment*. The idea is that the more precisely and accurately a patient is diagnosed, which secondarily enlists categorization, the better the treatment will be and the greater the likelihood of a positive outcome.

Given these ideas we can see why assigning percentages to classification is less important—and less precise.

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[444] Son, Lee, and Hwang, "Disease Severity Classification and COVID-19 Outcomes."

In the brief remarks that follow six classification labels are covered under three groupings. The most attention is paid to the third group in line with the logic outlined above.

Asymptomatic and Presymptomatic Cases

The name says it all: *asymptomatic* cases are 'without' (*a-*) 'symptoms' (*symptomatic*). Formally this is termed 'asymptomatic carriage' and infected persons of this kind are called 'asymptomatic carriers.' Typically, people infected with SARS-CoV-2 experience a brief period symptom-free before becoming symptomatic—called *presymptomatic*—and that label is sometimes grouped with asymptomatic so that 'asymptomatic/presymptomatic' includes anyone who is infected without symptoms at the time of classification. Some people never experience any noticeable symptoms and are therefore termed asymptomatic.

As noted above, an early study in the pandemic, looking at 72,314 cases in China, reported a rate of asymptomatic cases of just 1% (based on clinical observation of no typical symptoms but with a positive nucleic acid amplification test (NAAT)).[445] However, establishing an agreed upon estimate has proved elusive, with subsequent reports ranging from 4–90% because of variances in terminology and conception. Meta-analyses to estimate pooled asymptomaticity have found 16–20%, but have been criticized as having a downward bias.[446] Studies can raise confusion because some researchers report percentages of tested individuals and others report studies of confirmed COVID-19.

One systematic review and meta-analysis of 61 studies (18 using antigen tests; 43 using PCR tests) found at least a third of infections are asymptomatic and that of those without symptoms at the time of testing, nearly three-quarters remained symptom free.[447] A later systematic review and meta-analysis, this one covering 95 studies involving 29,776,306 individuals, reported a pooled percentage of asymptomatic infections among tested people of just 0.25%. Using 77 studies, among confirmed cases of COVID-19 (confirmed by RT-PCR test) the pooled percentage was 40.5%.[448] Another systematic review and meta-analysis of 390 studies used 170 of them to calculate asymptomaticity as 35.1%[449] The CDC's 'best estimate' in mid-March of 2021 was 30% of infections were asymptomatic.[450] An early study of Omicron suggested asymptomatic carrier spread might be a significant factor in the variant's rapid spread.[451]

One interesting footnote to this matter comes from a meta-analysis mentioned above, published in 2021. It found what the authors termed "a statistical-

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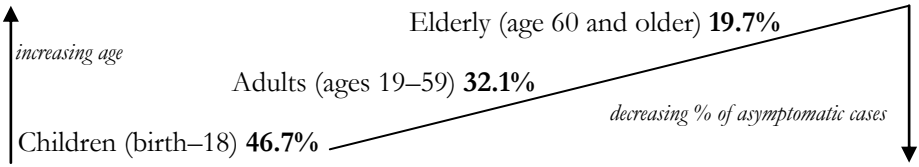
[445] Wu and McGoogan, "Characteristics of and Important Lessons," 1239.
[446] Sah et al., "Asymptomatic SARS-CoV-2 Infection," 1.
[447] Oran and Topol, "The Proportion of SARS-CoV-2 Infections that Are Asymptomatic." This study was criticized for its reliance on some studies in preprint status.
[448] Ma et al., "Global Percentage of Asymptomatic SARS-CoV-2 Infections," 2, 4.
[449] Sah et al., "Asymptomatic SARS-CoV-2 Infection," 3.
[450] CDC, "COVID-19 Planning Scenarios" (Mar. 19, 2021 update). Their best estimate of infectiousness of the asymptomatic relative to the symptomatic was 75%.
[451] Garrett et al., "High Rate of Asymptomatic Carriage."

ly significant trend toward a lower asymptomatic percentage with increasing age." It looks like this:[452]



The matter of how many people infected by SARS-CoV-2 or who are diagnosed with COVID-19 but remain asymptomatic is one—like so many others with this pandemic—still being studied.

Mild and Moderate Cases

As seen earlier, 'mild' and 'moderate' are sometimes grouped together under the label 'mild.' The line between asymptomatic and mild cases is sharper and more obvious than perhaps that between mild and moderate cases of COVID-19. The NIH and many others distinguish mild from moderate by technical measures with an emphasis on respiratory function. Many such patients recover with home care, but some eventually require hospitalization if symptoms worsen.

Early in the pandemic, examining cases in China through February 11, 2020, of 44,415 cases classified, 81% were labeled 'mild' (symptomatic with either no pneumonia or mild pneumonia).[453] Paul Auwaerter, Clinical Director, Division of Infectious Diseases, Johns Hopkins University School of Medicine, in early 2022 for the University's Guide to COVID-19 uses the figure of 'about 80%' for infections by SARS-CoV-2 that do not rise to the level of being severe and notes that this means patients recover without special treatment.[454] Some patients with moderate COVID-19 may be admitted to the hospital because of underlying conditions or other matters warranting closer observation and/or treatment.

Severe and Critical Cases

A critical line exists between mild and moderate cases on one side and severe cases on the other. Simply being admitted to hospital is not sufficient to indicate a severe case of COVID-19. However, it is estimated that *about 1-in-5 (20%) of hospitalized COVID-19 patients develop severe clinical complications* (e.g., acute respiratory distress syndrome (ARDS), systemic hyperinflammatory response, and/or death).[455] The early Chinese study referred to above, which distinguishes 'severe' and 'critical,' classified 19% in these two classes (14% 'severe' and 5% 'critical').[456] In Europe, as reported to the European Surveillance System (TES-

[452] Illustration based on Sah et al., "Asymptomatic SARS-CoV-2 Infection," 3–4.
[453] Wu and McGoogan, "Characteristics of and Important Lessons," 1239.
[454] Auwaerter, "Coronavirus COVID-19 (SARS-CoV-2)" (Jan. 14, 2022 update).
[455] Fagyas et al., "Circulating ACE2 Activity Predicts Mortality and Disease Severity," 8.
[456] Wu and McGoogan, "Characteristics of and Important Lessons," 1239.

Sy) for the span August, 2020–May, 2021, just 13.6% of hospitalized COVID-19 patients had severe enough cases (i.e., were 'critically ill') to warrant admission to an Intensive Care Unit (ICU).[457]

An International Severe Acute Respiratory and Emerging Infections Consortium (ISARIC) report covering 60,109 hospitalized patients across 43 countries found the three most common symptoms at admission were:

Symptom	Percentage of Patients
history of fever	68.7%
cough	68.5%
shortness of breath	65.8%

It is worth noting that 92% of patients admitted to hospital reported one or more of these.[458]

Standard Measures of Severe COVID-19

As noted in the classification materials above, some key measures have been adopted to identify severe cases of COVID-19. These are:

- *abnormally low concentration of oxygen in the blood* (*hypoxemia*)—determined as oxygen saturation (SaO2, or O2 Sat) ≤93%;
- *abnormally low arterial blood oxygen level*—determined as the ratio of partial pressure of arterial oxygen to fraction of inspired oxygen (P/F ratio, or more formally, PaO2/FiO2 ratio) <300 mmHg);
- *abnormally rapid breathing* (*tachypnea*)—determined as a respiratory rate ≥30 breaths per minute; and,
- *abnormal or foreign substances* (*infiltrates*) *accumulating within lung tissue*—determined as lung infiltrates >50%.

The first two probably require some distinction. Both are measures of hypoxemia—abnormally low levels of oxygen in blood. The first can be measured by a *pulse oximeter*—a small device that simply clips on to a finger or earlobe. It provides a quick reading. Normal blood oxygen levels are 95–100%. The second measurement method complements the first without reproducing it. Where the first—SaO2—measures the percent of Hemoglobin (Hgb) binding sites in the blood that are carrying oxygen, the second—PaO2/FiO2 ratio (or simply P/F ratio)—measures the actual oxygen content in arterial blood. At sea level the normal P/F ratio is ~ 400-500 millimeters of mercury (mmHg). Measurement requires drawing and testing an arterial blood sample, which is more time consuming. Using both is a sensible way to confirm a potential problem. The problem is Acute Respiratory Distress Syndrome (ARDS).[459]

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[457] European Centre for Disease Prevention and Control (ECDC), "Clinical Characteristics of COVID-19)" (Sept. 7, 2021 update).

[458] ECDC, "Clinical Characteristics of COVID-19)" (Sept. 7, 2021 update).

[459] For more on this matter, see Lai et al., "The Ratio of Partial Pressure Arterial Oxygen."

The last two also may be remarked upon together. If we look back at the NIH material earlier in this section, we see that these measures are joined together so that one or the other suffices; both together are not required. Lung infiltrates can lead to rapid breathing. Each can suggest the presence of pneumonia.[460]

Biomarkers

Four classes of *biomarkers*—measurable biological signs—have been suggested as providing a pattern associated with severe cases:

- *blood (hematologic) biomarkers*—these include white blood cell (WBC) counts (especially for lymphocytes and neutrophils);
- *biochemical biomarkers*—these include those related to red blood cell breakdown (*bilirubin* tests), to cardiac muscle enzyme (CK), a liver enzyme (AST), etc.;
- *inflammatory biomarkers*—these include measuring processes underlying inflammation of blood vessels (*vasculitis*); and,
- *coagulation biomarkers*—these include looking for elevated levels of certain biomarkers (FDP and D-dimer).[461]

White blood cell counts (WBC) showing severe depletion of lymphocytes is termed *lymphocytopenia*, a condition associated with severe COVID-19 and accompanied by a decrease in other kinds of WBCs (monocytes and eosinophils). At the same time, there is an increase in another kind of WBC—neutrophils—leading to an increased neutrophil (NEU)-to-lymphocyte (LYM) ratio (NLR).[462]

The biochemical biomarkers mentioned focus on factors related to blood clots (specifically *thromboembolisms*), to heart (cardiac) injury, and to liver damage (increased AST levels). The inflammatory biomarkers include tests of the *C-Reactive Protein* (CRP), looking for elevated levels indicating inflammation. Elevated levels of certain substances are also keys to assessing coagulation biomarkers. Both *fibrin degradation products* (FDP) tests and tests of *D-dimer* levels are used to gauge blood clotting. Both FDP and D-dimers are fragments produced in the clotting process. One study published in 2020 reported that D-dimer was the only variable at admission to hospital that was associated with increased odds of mortality.[463]

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[460] Bahrami-Matlagh et al., "Agreement between Lose-Dose and Ultra-Low-Dose Chest CT," 7, report that "ultra-low-dose chest CT scan had a very good diagnostic performance for diagnosis of lung infiltration suggestive for viral pneumonia during the COVID-19 pandemic compared to low-dose CTscan with a sensitivity of 100% and specificity of 98.4%."

[461] Ponti et al., "Biomarkers Associated with COVID-19 Disease Progression," 391–95.

[462] Ponti et al., "Biomarkers Associated with COVID-19 Disease Progression," 392.

[463] Yao et al., "D-dimer as a Biomarker for Disease Severity."

In addition to empirical testing of biomarkers, doctors rely on their training, experience, and skills to use *clinically observed conditions*—'symptoms'—to gauge the severity of COVID-19 illness. The following illustration shows the role of key clinical symptoms alongside diagnostic testing and epidemiological investigation (e.g., determining recent travel history and exposure):

Suthar et al., "Coronavirus Disease Case Definitions, Diagnostic Testing Criteria, and Surveillance in 25 Countries with Highest Reported Case Counts." *Emerging Infectious Diseases* 28/1 (Jan., 2022) 148-156. Table from p. 150.

Table 1. Selected suspected case definition criteria across 25 countries with the highest COVID-19 case counts, current as of January 1, 2021*

		Clinical symptoms								
Country	Diagnostic testing/laboratory evidence†	Cough	Fever	SARI	Labored breathing	Headache	Muscle pain	Loss of taste or smell	Diarrhea	Epidemiologic criteria, any
WHO definition (reference)		X	X	X	X	X	X		X	X
Argentina		X	X	X	X	X	X	X	X	X
Bangladesh‡		X	X	X	X	X	X		X	X
Brazil		X	X	X	X	X		X	X	
Chile		X	X	X	X	X	X	X	X	X
Colombia	X	X	X		X	X	X	X	X	X
France§¶		X	X		X	X	X	X	X	
Germany§¶		X	X		X	X	X	X	X	
India#		X	X	X	X					X
Indonesia			X	X	X					X
Iran			X	X						X
Iraq‡		X	X	X	X	X	X		X	X
Israel**										X
Italy§¶		X	X		X	X	X	X	X	
Mexico		X	X		X	X	X	X		
Pakistan#		X	X	X	X					X
Peru		X	X	X	X	X				
Philippines‡		X	X	X	X	X	X		X	X
Russia		X	X		X		X	X	X	
Saudi Arabia		X	X	X	X	X		X	X	X
South Africa		X	X	X	X		X	X	X	X
Spain		X	X	X	X	X	X	X	X	
Turkey		X	X	X	X	X	X	X	X	X
Ukraine		X	X	X	X					X
United Kingdom¶		X	X					X		
United States	X									
No. (%) countries including criterion††	2 (8)	21 (84)	23 (92)	16 (64)	21 (84)	15 (60)	14 (56)	14 (56)	15 (60)	16 (64)

*Complete data are available in Appendix Table 3 (https://wwwnc.cdc.gov/EID/article/28/1/21-1082-App1.pdf). X indicates the criterion was sufficient for, or a potential component of, the suspected case definition requirement(s). COVID-19, coronavirus disease; SARI, severe acute respiratory infection; WHO, World Health Organization.
†See suspected case definition for applicable country (Appendix Table 2).
‡World Health Organization definition (updated August 2020).
§European Centre for Disease Prevention and Control definition.
¶These countries consider these definitions as possible not suspected cases; because of the comparability between possible and suspected, we treated these definitions as a suspected definition.
#World Health Organization definition (updated March 2020).
**Israel does not have an official suspect case definition; persons are considered suspected on the basis of contact with confirmed cases determined by digital surveillance of cellphones.
††Denominator is 24 countries with suspected case definition.

This open access public domain material is available online at https://wwwnc.cdc.gov/eid/article28/1/21-1082_article
Authors: Amitabh B. Suthar, Sara Schuubert, Julie Garon, Alexia Couture, Amy M. Brown, and Sana Charania.

One team of researchers have proposed a ridge regression model—a statistical method employed in situations where the number of observations available is exceeded by the number of predictor variables in a set. In their model they derived eight variables to help assess patients specifically with the likelihood of the patient developing severe COVID-19. As can be seen when looking at their variables, they combine the bio-markers and clinical observations discussed above. Their eight variables are (in the order they list them):

- *lactate dehydrogenase* (LDH) test—which looks for increased levels suggestive of tissue damage (e.g., in blood, brain, heart, kidney, or lungs);
- *C-reactive protein* (CRP) test—which looks for elevated levels suggestive of inflammation;
- *albumin blood* (ALB) test—which looks for decreased levels suggestive of kidney or liver problems;
- *comorbidity*—the presence of underlying medical conditions (such as discussed earlier in the chapter);
- *electrolyte disturbance*—an imbalance in electrolytes (either too high or too low) suggesting dysregulation in one or another system (e.g., cardiac, digestive, muscular or nervous system);
- *coagulation function*—which assesses blood clotting in the body;
- *eosinophil test*—which measures the amount of this kind of white blood cell to gauge response to an infection; and,
- *lymphocyte counts*—another measure of a type of white blood cell used to gauge response to an infection.

Using these variables in their model the researchers were able with a modestly sized group of patients to determine *risk score medians* for each of four classes of COVID-19:

	Mild	**Moderate**	**Severe**	**Critical**
Risk Score Median	9.19	45.65	102.38	113.42

It doesn't require understanding exactly what these scores means to see at once there are apparently significant differences of magnitude. In their study, which they point out was with a limited number of patients, the most frequent classification of a COVID-19 patient was as having a 'moderate' case, followed by 'mild,' and 'severe' cases.[464]

Moderate cases may be admitted to hospital on the basis of symptoms and/or over concern of becoming severe. Quite understandably, because severe cases represent poorer outcomes, including serious body trauma and death, they receive the most attention. The accurate assessment of severe cases is essential to effective treatment, which is why so many different factors have been considered as physicians and scientists try to provide life-saving information.

It is beyond the reach of this volume to examine all of the factors that have been researched in the hope of better understanding disease progression in COVID-19 and the factors that predict or are associated with disease severity. However, in this regard certain matters have surfaced as particularly important.

[464] Qi et al., "Severity-Associated Markers and Assessment Model," esp. 5–6, for variables and risk score medians.

Notable Conditions Associated with Severe and Critically Ill Cases

There are certain things that especially draw attention in severe cases of COVID-19. They include:

- difficulty breathing (*dyspnea*);
- abnormally low concentrations of oxygen in the blood (*hypoxemia*);
- abnormally low levels of lymphocytes (*lymphopenia*);
- the development of *acute respiratory distress syndrome* (ARDS); and,
- the role of *inflammation*, especially systemic hyperinflammatory response.

Dyspnea

Dyspnea is a term we have encountered fairly often. The Latin term is transliterated from Greek (δύσπνοια) and means literally 'bad' (*dys-*) 'breathing' (*-pnea*), which is generally translated in medical circles as either 'difficult' breathing (i.e., difficulty in breathing), or 'labored' breathing; in common parlance it often refers to 'shortness of breath' and has sometimes been styled as 'air hunger.' Physiologically, dyspnea is a subjective 'sensation.'[465] It has been reported as the most common symptom of severe COVID-19.[466]

Dyspnea is most often thought to result from a mismatch between motor output from the respiratory center and the afferent input from nerve receptors—when the information from output and input don't meet in agreement in the cerebral cortex, dyspnea results.[467] It is a critical factor even among hospitalized COVID-19 patients without pre-existing comorbidities (underlying health conditions). A study of 4,806 patients (median age: 47; median length of illness from symptom onset to discharge or death: 32 days) found that dyspnea (15.33%) was second as an initial symptom only to fever (75%). It consistently emerged as a risk factor related to progression from severity on admission to the hospital to death while there.[468]

Dyspnea is also a feature of *viral myocarditis*—inflammation of the heart muscle—and it has been remarked that its symptomatology and that of COVID-19 are "almost indistinguishable." A systematic review of the literature conducted by one group of researchers uncovered 51 cases of COVID-19-associated myocarditis. Dyspnea was present in 79% of these cases—a rate higher than for all COVID-19 patients (16.4%), and those with severe COVID-19 requiring intensive care (53.7%). The researchers further note that, unlike in viral myocarditis

[465] Hirota et al., "Asymptomatic Hypoxemia," 51–52.
[466] Berlin, Gulick, and Martinez, "Severe COVID-19," 2451.
[467] This *output-reinput mismatch* or *central-peripheral mismatch* theory is succinctly explained in Hirota et al., "Asymptomatic Hypoxemia,"53.
[468] Liu et al., "Risk Factors for COVID-19 Progression and Mortality."

without COVID-19, in COVID-19 dyspnea typically is secondary to multifocal pneumonia (i.e., pneumonia in different locations in the lungs), and ARDS.[469]

Hypoxemia

Hypoxemia—abnormally low concentrations of oxygen in the blood (oxygen saturation (SaO_2, or O_2 Sat) ≤93%)—we saw earlier plays an especially prominent role in diagnosing a case of COVID-19 as severe. When oxygen saturation (as SpO_2) falls to 90% or less (as in pneumonia), the lungs' capacity to inflate is severely affected, interfering with the proper exchange of gasses such that carbon dioxide (CO_2) levels increase and the person experiences drowsiness and/or dyspnea.[470] It is not only closely associated with pneumonia but especially with ARDS, where rapidly progressive hypoxemia leads to high mortality rates.[471] A study published in 2020 of 140 patients with COVID-19-associated pneumonia a SpO_2 cutoff value of 90.5% was highly predictive of mortality. Increasing oxygenation by even modest degrees (1-U) reduced mortality risk by 8%.[472]

Hypoxemia often accompanies dyspnea.[473] However, the percentage of COVID-19 patients with both is only about 30% when the range of patients is expanded to include mild cases. Among severe cases, 60–70% complain of difficulty breathing.[474]

But hypoxemia associated with COVID-19 is not all of one kind. Hypoxemia is expected to produce elevated levels of CO_2, with the poor oxygenation leading to labored breathing. But in so-called *happy hypoxemia* (or *silent hypoxemia*, or *asymptomatic hypoxemia*) neither labored breathing, nor elevated CO_2 levels, are evident. The patient in these respects seems normal.[475] The hidden danger, though, is that this presentation of hypoxemia also can lead to ARDS. Estimates of how common hypoxemia is among COVID-19 patients have not been firmly fixed, but one retrospective cohort study of 270 such patients found 3% met the criteria for asymptomatic hypoxemia.[476]

Severity of hypoxemia has been significantly associated with *interleukin 6* (IL-6)—a kind of small protein known as a *cytokine*, an important part of the immune system.[477] In COVID-19 increased levels of IL-6 in blood serum (>35 pg/mL) are correlated with more severe pneumonia, increased need for mechanical ventilation, and an increased risk of death.[478]

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[469] Hausner et al., "COVID-19 Associated Myocarditis," 152.
[470] Nair et al., "Happy Hypoxemia," 066.
[471] Donina, "Causes of Hypoxemia in COVID-19."
[472] Xie et al., "Association between Hypoxemia and Mortality." See especially p. 1143–44.
[473] Berlin, Gulick, and Martinez, "Severe COVID-19," 2451. But cf. Hirota et al., "Asymptomatic Hypoxemia," 49.
[474] Hirota et al., "Asymptomatic Hypoxemia," 56.
[475] Nair et al., "Happy Hypoxemia," 066.
[476] Hirota et al., "Asymptomatic Hypoxemia," 56.
[477] Leatherdale et al., "Persistently Elevated Complement Alternative Pathway Biomarkers," 10.
[478] Guirao et al., "High Serum IL-6 Values."

Lymphopenia (literally, 'a poverty of lymph cells (*lymphocytes*)) is an abnormally low number of lymphocytes in the blood. Lymphocytes—a type of white blood cell—are of two main kinds: antibody-producing B-cells and T-cells capable of the extreme step of killing the body's own cells when overwhelmed by viral material. While there seems not to be a universally agreed upon number for establishing lymphopenia (because 'normal' levels vary by age and other factors), various studies have established as a cut-off point between 'normal' and 'abnormally low' figures ranging between ≤ 1100–≤ 1500 microliters (μL).[479]

Lymphopenia has been said to occur in a majority of severe cases of COVID-19.[480] Earlier we saw it was found in 46% of women and neonates studied with respect to COVID-19 and pregnancy.[481] Lymphopenia has been implicated as a predictor of disease severity among those who have it *before* infection by SARS-CoV-2.[482] One study in early 2020 found blood lymphocyte percentage (LYM%) the most consistent predictor of disease progression to death, with reductions to lower than 5% within two weeks of COVID-19 onset among most of those who died. Conversely, those who were severe cases but recovered showed initial decline followed by an increase in LYM%, while moderate cases showed little change.[483]

A systematic review and meta-analysis of 23 studies, involving 3,099 patients, reported progressively greater mean differences as the patients' conditions grew more dire:

Patient Status	**Mean Difference** **(from Patients with Better Outcomes)**
Severe COVID-19	−353.34 μL
Intensive Care Unit (ICU) Admission	−376.53 μL
Development of ARDS	−377.56 μL
Died of COVID-19	−395.35 μL

The researchers found a mean difference of −361.06 μL in lymphocyte counts between those COVID-19 patients who had a 'good outcome' and those who had a 'poor outcome.' [484]

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[479] Huang and Pranata, "Lymphopenia in Severe Coronavirus Disease-2019," 5. Note: the National Heart, Lung, and Blood Institute has an expansive range of normality (1,000–4,000 μL).
[480] Berlin, Gulick, and Martinez, "Severe COVID-19," 2451.
[481] Dubey et al., "Maternal and Neonatal Characteristics," 394.
[482] Garbo et al., "Pre-Existing Lymphoneia Increases the Risk of Hospitalization."
[483] Tan et al., "Lymphopenia Predicts Disease Severity," 1.
[484] Table consturcted from data in Huang and Pranata, "Lymphopenia in Severe Coronavirus Disease-2019," 3.

Acute Respiratory Distress Syndrome (ARDS)—by name alone a hallmark condition in a disease named after Severe Acute Respiratory Syndrome (SARS) Coronavirus (CoV) 2—is a serious medical condition in which fluid buildup in the lung's air sacs (*aveoli*) hinders oxygen reaching the body's organs. Typically rapid in progression ARDS is associated with severe shortness of breath and often leads to death.

The condition has been known by this name since the late 1960s, but was ambiguously defined until 1994 when the American-European Consensus Conference (AECC) specified four criteria covering:

- *acute onset*—the timing is characterized by rapid development;
- *oxygenation threshold* (hypoxemia criterion)—$PaO_2/FiO_2 \leq 300$ millimeters of mercury (mmHg), regardless of Positive End-Expiratory Pressure (PEEP);
- *bilateral infiltrates*—abnormal or foreign substances (infiltrates) affecting both sides (bilateral) of the lungs, as seen on x-ray (frontal chest radiograph); and,
- *mean airway pressure* (PAW) $\leq$ 18 mmHg when measured, or no clinical evidence of left arterial hypertension (i.e., high blood pressure in that artery).[485]

Though what the AECC set forth has been widely used to good effect, it has proved imperfect, with some imprecision lingering (e.g., does 'acute' mean within hours, days, weeks?). As a result, some two decades later, the so-called 'Berlin Definition' (2012) proposed distinguishing three categories (mild, moderate, severe) for ARDS, specified a timeframe of one week for 'acute,' and tightened expectations for interpreting chest x-rays.[486]

Early on researchers sought how to characterize ARDS as seen in COVID-19. One team of researchers observed that severe COVID-19's manifestations can be seen as a combination of viral pneumonia and ARDS. Using the Berlin definition and its criteria, they reported ARDS developing in 42% of patients presenting with COVID-19 pneumonia, with 61–81% of those requiring intensive care. They found a median time of 8.5 days to intubation after symptom onset, with mortality ranging from 26–61.5% after ICU admission and 65.7–94% among those receiving mechanical ventilation. They noted that COVID-19 ARDS seemed associated with worse outcomes than typical ARDS.[487]

The quest to understand COVID-19-associated ARDS continues. Some speculation has been made that it is of two phenotypes (type H and type L), sharing similar levels of hypoxemia, but differing in other important respects

[485] Bernard et al., "Report of the American-European Consensus Conference," 226–27.
[486] Ferguson et al., "The Berlin Definition of ARDS."
[487] Gibson, Qin, and Puah, "COVID-19 Acute Respiratory Distress Syndrome (ARDS)."

(e.g., CT imaging).[488] Type H has been called 'typical ARDS' and type L termed 'non-ARDS).[489] This kind of reality can complicate assessment and treatment, but anyway it is looked at ARDS is a serious matter; though rare overall, it can be quickly deadly when it does develop.

A retrospective study of 116,539 COVID-19 cases in Poland for the period March–December, 2020, reported an overall prevalence of ARDS of 3.6%, with it being more likely among males (4.4%) than females (2.9%). Among those hospitalized with COVID-19-related ARDS the mortality rate was 88%—a higher mortality rate than that reported in most other countries. The researchers' examination of the global literature on the subject revealed that about one-third (33%) of hospitalized COVID-19 patients develop ARDS and a meta-analysis of 10,185 COVID-19 ARDS cases in seven countries showed a pooled mortality estimate of 39%.[490]

Respiratory problems can be addressed in different ways. Outside intensive care units *continuous positive airway pressure* (CPAP) machines are sometimes used. CPAP might be delivered via helmets, face masks, or flow meter. Such patients can be candidates for intubation if the CPAP procedure fails. In that respect, research has indicated risk increases by sex (male), high lactate dehydrogenase (LDH) levels, and too low a ratio of arterial partial pressure of oxygen to fraction of inspired oxygen ($PaO_2/FiO_2 < 200$ mmHg, measured 1 hour after noninvasive positive-pressure ventilation (NPPV)).[491] Put in more common terms, more men than women need intubation and two biological markers—high LDH levels and low P/F ratio—indicate increased risk of intubation. The first marker, high LDH levels, points to possible tissue damage, with very high levels perhaps marking multiple organ failure. Low P/F ratio (PaO_2/FiO_2) compares the concentration of oxygen breathed in to that actually found in the blood. The cut-off point of 200 mmHg is about half of what is considered normal for adults.

As mentioned earlier in the chapter, endotracheal intubation and invasive mechanical ventilation (IMV)—a procedure by which a machine ventilator pumps air through a tube down the throat into the lungs to assist breathing—is sometimes used. *Rapid sequence induction and intubation* (RSII)[492] is the airway management technique commonly employed with patients at risk for pulmonary aspiration, using various drugs and with some modifications from place to place.[493]

Intubation may be indicated for COVID-19 patients when a loss of consciousness occurs, there is hypoxemia, or ARDS is present. But how frequently

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[488] Gando and Wada, "Pathomechanisms Underlying Hypoxemia," 1.
[489] Huang et al., "The Role of Extracorporeal Membrane Oxygenation," 1.
[490] Gujski et al., "The Prevalence of Acute Respiratory Distress Syndrome (ARDS)."
[491] De Vita et al., "Predictors of Intubation in COVID-19 Patients," 5.
[492] RSII is commonly shortened to just RSI when the obvious subject matter is intubation. Occasionally the label *rapid sequence intubation* is used, and sometimes as distinct from 'rapid sequence induction,' but see Ghafoor and Martin, "'RSI.'"
[493] For a general introduction to the procedure see El-Orbany and Connolly, "Rapid Sequence Induction and Intubation Current Controversy."

it is employed varies widely based on a number of factors. A review of 24 studies covering seven countries (Brazil, China, Iran, Italy, South Korea, Spain, and the US), found wildly varying rates of intubation (5–88%).[494] A systematic review and meta-analysis of a dozen studies of critically ill COVID-19 patients examined what role timing of intubation might make. It found no effect on morbidity or mortality whether initiated early or late—a finding they suggest might justify more often embracing a 'wait-and-see' approach.[495]

Intubation and IMV often precede another treatment step. Common to the treatment of COVID-19-related ARDS, though often regarded as a step of last resort, has been *extracorporeal membrane oxygenation* (ECMO). Sometimes referred to as 'extracorporeal life support,' ECMO involves pumping blood from the body to a heart-lung machine. There the proper balance of oxygen is restored to the blood and it is returned to the body. One important aspect of this method is that it provides some healing rest to the heart and lungs as the machine does the work they normally conduct. There are two types of ECMO: VA ECMO has a connection to both a vein (V) and an artery (A); VV ECMO is vein-to-vein (VV). In cases of severe COVID-19-related ARDS, VV ECMO is more commonly used, though VA ECMO is sometimes employed in patients with circulatory disorders. Variations in outcome, researchers suggest, point to the importance of selecting this method for appropriately selected patients.[496]

Just as ARDS has associations with dyspnea and hypoxemia, so it also has connections with inflammation—our next issue of concern. A close interplay between coagulation and inflammation in acute lung injuries such as seen in ARDS has been long established by research.[497]

Inflammation

Inflammation—a normal protective reaction in the body—is a critical issue. Through this mechanism the body seeks to arrest the viral infection by eliminating the viral material. Severely damaged cells are destroyed. Incited by white blood cells, inflammation raises the blood flow to an affected area. A person experiences inflammation in a variety of ways, through sensations of heat, swelling, stiffness and soreness, and so forth. Fever is a marker of inflammation.

The inflammatory responses triggered by COVID-19 have been called "unique"; they point to severe disturbance in immune response.[498] Immunologist Jose Guirao and colleagues succinctly summarize the importance of this

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[494] Mohammadi, Khamseh, and Varpaei, "Invasive Airway 'Intubation' in COVID-19," 1.

[495] Papoutsi et al., "Effect of Timing of Intubation."

[496] Huang et al., "The Role of Extracorporeal Membrane Oxygenation." They observe, 8, that early initiation of ECMO, especially among younger patients, can reduce mortality significantly (e.g., from 65% to 45% in cases of younger patients without severe comorbidities when initiated within seven days of tracheal intubation).

[497] Gando and Wada, "Pathomechanisms Underlying Hypoxemia," 1, citing Welty-Wolf et al., "Coagulation and Inflammation in Acute Lung Injury," who discuss the matter in connection to progressive ARDS.

[498] Hirota et al., "Asymptomatic Hypoxemia," 54.

matter: "The inflammatory response plays a critical role in COVID-19, and inflammatory cytokine storm increases the severity of COVID-19."[499]

SARS-CoV-2 is a *cytopathic* virus—it 'harms' (*-pathic*) 'cells' (*cyto-*). The host immune response at the site of infection is to employ the biological tools discussed in the next chapter. Here we will stay very simple. Inflammatory responses may vary somewhat by location in the body, but they share a common mechanism of action with these characteristics:

- *pattern receptors* on the affected cell recognize the infectious agent;
- inflammatory *pathways* to the site are activated;
- inflammatory *markers* are released; and,
- inflammatory *cells* are recruited to action.

Linlin Chen and colleagues, who set out the above characteristics, note succinctly, "The inflammatory response involves a highly coordinated network of many cell types."[500]

Most of the time the body's inflammatory response is highly effective and the infection is cleared; the person recovers as the immune response recedes. However, in severe COVID-19 a dysfunctional immune response sometimes occurs. This releases a *cytokine storm*—cytokines being proteins secreted in inflammation that are important in modulating the inflammation response. In a cytokine storm what begins as a local site of inflammation spreads throughout the body through overproduction of both inflammatory cytokines and chemokines (small proteins that stimulate migration of cells such as white blood cells (leukocytes)), with these being released not just by immune system cells but by other kinds of cells as well (e.g., endothelial cells, smooth muscle cells, and fibroblasts).[501]

In the lungs—particularly vulnerable to it—cytokine storm results in severe pathology. It is considered a leading cause of ARDS and multi-organ failure, thus it and its associated complications are a major cause of death in COVID-19 patients.[502] In addition to the above, inflammation through cytokine storm also might be linked to cardiac injury, acute kidney injury, liver injury, gastrointestinal injury, neurological injuries, and so forth.[503] So critical is this situation when it occurs that one team of researchers concluded their review of the matter by remarking, "Controlling the inflammatory response may be as important as targeting the virus."[504]

One line of thinking looks at the SARS-CoV-2 nucleocapsid (N) protein—the covering over the viral RNA—and its possible role in more advanced cases

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[499] Guirao et al., "High Serum IL-6 Values," 66.
[500] Chen et al., "Inflammatory Responses," 7205 (list), and 7207 (quote)..
[501] Iwasaki et al., "Inflammation Triggered by SARS-CoV-2," 17.
[502] Iwasaki et al., "Inflammation Triggered by SARS-CoV-2," 18. Also see Kunnumakkara et al., "COVID-19, Cytokines, Inflammation, and Spices," 4–5.
[503] Iwasaki et al., "Inflammation Triggered by SARS-CoV-2," 21–23.
[504] Tay et al., "The Trinity of COVID-19," 366 and 372 (quote).

of the disease. This protein may bind to a region in certain host cells that are involved in inflammatory processes in the body. Specifically, the N protein binding to the *prostaglandin-endoperoxide synthase 2 (PTGS2) / cyclooxygenase-2* (COX-2) *promoter* can increase ('upregulate') expression of the gene which codes the COX-2 enzyme that propels production of the *prostaglandin E2* (PGE2)—a lipid chemical messenger made at sites of infection—and certain other molecular agents that promote inflammation. This inflammation is part of the body's natural defense against infection, but runaway inflammation is both unpleasant and dangerous. Elevated levels of PGE2, for example, may drive hyper-activation of *mast cells*—a kind of cell in connective tissue that is a key player in the body's inflammatory response by releasing histamine and other chemicals. In hyper-activation of these cells there is excess release of histamine and other inflammatory agents. This course of disease progression would, at least in some cases, make COVID-19 a mast cell disease.[505]

What this means is that the just described course of action might be behind the increased histamine levels some older children and some adults experience with COVID-19. Most people are familiar with histamines in connection with allergic reactions. Histamines are part of the body's response to antigens, which is good, but too much of a good thing is bad. Elevated histamine levels can result in serious heart issues.[506]

Platelet-to-lymphocyte count ratio (PLR) has emerged as a prognostic predictor with COVID-19 patients. This is because it has become clearer that platelets and lymphocytes can be associated with inflammatory responses. Elevated PLRs are strongly associated with systemic inflammation.[507] PLRs can be calculated from a blood sample drawn from the patient, where a complete blood count is done, meaning that it is a relatively convenient biomarker to assess. With specific respect to COVID-19, a systematic review and meta-analysis of 32 studies found that a higher PLR on hospital admission was associated with more severe illness and increased risk of death.[508]

Final Note

Severe cases of COVID-19 are especially associated medically with pneumonia, Acute Respiratory Distress Syndrome (ARDS), and organ failure. But in popular perception they are most associated with two words: hospitalization and death. Among those most severely ill—the critically ill—the mortality rate hovers around half (49%).[509] It is time to focus on the issue of mortality.

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[505] Ricke, "Two Different Antibody-Dependent Enhancement (ADE) Risks," 2.
[506] Ricke, "Two Different Antibody-Dependent Enhancement (ADE) Risks," 5.
[507] Shen, Haung, and Zhang, "Platelet-to-Lymphocyte Ratio," 5.
[508] Sarkar et al. "Role of Platelet-to-Lymphocyte Count Ratio (PLR)."
[509] Wu and McGoogan, "Characteristics of and Important Lessons," 1239; also Berlin, Gulick, and Martinez, "Severe COVID-19," 2452.

Mortality

The COVID-19 pandemic has resulted in significant loss of life around the world. By the end of the first week of January, 2022, more than 296 million confirmed cases with nearly 5.5 million dead.[510] The loss of life worldwide in the first six months of 2021 exceeded that for all of 2020.[511]

Decreased Life Expectancy

One measure of the impact of the pandemic is the fact that in 2020 *life expectancy at birth declined in 27 of 29 countries* studied—and these nations included the U.S. and most European countries. Life expectancy declined by more than a year for males in 11 countries and more than a year for females in 8 countries. In much of Western Europe, the researches remarked, "the magnitudes of losses observed in 2020 have not been witnessed since World War II."[512]

Matters have been grim in the United States, too. Of the 29 nations included in the above study, *the U.S. had the largest loss of life expectancy for both males (more than 2 years) and females (more than 1.5 years)*.[513] Another study found that in the U.S. before the pandemic—during the period 2010–2018—life expectancy at birth increased (0.08 years). Between 2018–2020 life expectancy at birth decreased an estimated 1.87 years (2%). The decrease was proportionally greater at ages 25 (3.4%) and 65 (5.7%).[514]

Mortality Figures for the United States

As of December 15, 2021, there were 814,806 deaths attributed to COVID-19 in the United States. These distributed as follows (chronological order):[515]

Age Range	Number	Percentage (%)	Pooled %	
0–17	678	0.08%		
18–29	4,956	0.6%	0.7%	
30–39	14,614	1.8%		2.5%
40–49	35,190	4.3%	6.1%	
50–64	151,396	18.6%		
65–74	185,613	22.8%	41.4%	
75–84	209,701	25.7%		
85 and older	212,658	26.1%	51.8%	97.5%

[510] WHO, *WHO Coronavirus (COVID-19) Dashboard* (Jan. 6, 5:13 p.m. CET) reported 296,496,809 confirmed cases with 5,462,631 deaths (https://covid19.who.int/).
[511] Kamp, Douglas, and Forero, "COVID-19 Deaths."
[512] Aburto et al., "Quantifying Impacts of the COVID-19 Pandemic." The quote is from p. 6.
[513] Aburto et al., "Quantifying Impacts of the COVID-19 Pandemic," 9.
[514] Woolf, Masters, and Aron, "Effect of the Covid-19 Pandemic," 3.
[515] Age ranges and data from CDC (National Center for Health Statistics), "Weekly Updates by Select Demographic and Geographic Chracteristics," Table 1. Cf. Statista (https://www.statista.com/statistics/1191568/reported-deaths-from-covid-by-age-us/), which also compiles data.

COVID-19 rose from a small percentage of all deaths in the United States in January, 2020, to the leading cause of death in September, 2021. Mortality was associated with pandemic 'waves' and their variants. Overall, for the period 2020–2021, COVID-19 ranked as the third leading cause of death (behind heart disease and cancer).[516]

It is tempting, because of COVID-inspired anxiety, to focus on percentages, especially when one is younger, to try to calculate one's risk of death. It is easy to think, 'I am under 40 so my risk of dying from COVID-19 (even if I'm unlucky enough to get it) is extremely small. After all, less than 3% of people under age 40 die of the disease.' But that 2.5% represents 20,248 *actual persons*—children, brothers, sisters, aunts and uncles, fathers and mothers. They are not just numbers. It is also tempting, for the same reason of anxiety, to focus on the percentages for older ages, thinking, "I'm over 50, and 93% of those who die are over 50—I'm doomed!' But that misunderstands the statistics.

Simply calculating the percentage for a group of all those who died of COVID-19 doesn't establish a person's own risk of dying if they become infected. Deaths from COVID-19 typically entail one or more of the factors of vulnerability discussed earlier in the chapter. Even when statistics are looked at they need context. Such context includes not only the underlying factors contributing to mortality, but the nature of the statistics themselves. Let's consider two.

Case Fatality Ratio (CFR) and Infection Fatality Ratio (IFR)

Case fatality ratio (CFR) reports proportion of fatality based on confirmed cases; the number of people who have died from COVID-19 is divided by the total number of people diagnosed with confirmed cases for a given period of time, then multiplied by 100.[517] The table above does *not* report CFR, though it follows a similar manner of computation. The total number of people whose deaths were attributed to COVID-19 at a particular date (December 29) was 814,806. Each age range group is a subset of that larger number. Dividing the number of dead in an age range by the total COVID-19 dead (x100) provides the percentage of all dead that group represents. But it does *not* present CFR because it does not provide the total number of diagnosed cases of COVID-19.

A ratio like CFR is prone to change as the epidemic progresses. The number of confirmed cases grows, as does the number of deaths attributed to COVID-19 but the relation between these numbers can change because of any number of factors, such as a more deadly variant (Delta) or less deadly variant (Omicron) gaining ascendancy, the number of people accepting preventive care through vaccination, or better treatment being developed. Worldwide, the *reported case fatality ratio* (rCFR) for 2020 was 2.2% (based on 2.08 million deaths out of 96.9 million confirmed cases). Analysis showed that the rCFR peaked during the 17th epidemiological week (April 22–28) at 7.23%. Of course, there

[516] CDC, "Monthly Provisional Counts of Death by Select Causes, 2020–2021."
[517] WHO, "Estimating Mortality from COVID-19," 2.

were varying rCFRs around the world. For example, even among the five nations with the highest rCFR the numbers could differ dramatically: Yemen's rCFR was 28.9% while France was 11%.[518] In early 2022, the observed case fatality ratio for the United States was reported by Johns Hopkins University's Coronavirus Resource Center as about 1.5%. Neighboring nations had different rates—Canada was at 1.3%; Mexico stood at a CFR of 7.5%.[519]

Infection fatality ratio (IFR) is similar to CFR but harder to calculate. It is the proportion of fatalities based on total number of infections; the number of people who have died from COVID-19 is divided by the total number of people infected with SARS-CoV-2 for a given period of time, then multiplied by 100.[520] Of course, actually knowing the number of infected people—a number larger than the number of confirmed cases—is almost certainly impossible. Many infections are asymptomatic or go unconfirmed, while others are symptomatic but never reported. Complicating matters is that IFR and CFR can be treated the same simply by defining the number of *cases* as being the total number of *infections*—something possible in small, constrained situations but not in a pandemic, where the IFR can only be estimated.[521]

Omicron and Death

Some anti-vaxxers have been heard to proclaim that the Omicron variant does not cause death. That constitutes a nice fantasy, but that is precisely what it is—a fantasy. Even before the end of January, 2022, the daily number of deaths from Omicron had exceeded those from Delta when the latter variant was ascendant. In the latter half of that month the daily death average for COVID-19 in a week's span (Jan. 20–26) reached *2,271 deaths per day*[522] and projections were the number of deaths a day would continue to increase over the next few weeks.[523] This was happening because even though Omicron carries less risk of death it is so much more infectious that a high number of deaths still occur. It is easy enough to picture with a simple illustration: if 2 out of 100 people die from X, but only 2 out of 200 people dies from Y, if Y cases are twice as many as X cases then the number of dead is the same.

All of this was predictable—and, in fact, was predicted. The illustration that follows, courtesy of the Institute for Health Metrics Evaluation (IHME), an independent global health research center at the University of Washington, shows projections for daily deaths from COVID-19 as set out on the IHME website on January 11, 2022. The projection was made during the pandemic wave associated with Omicron's dominance in the United States.

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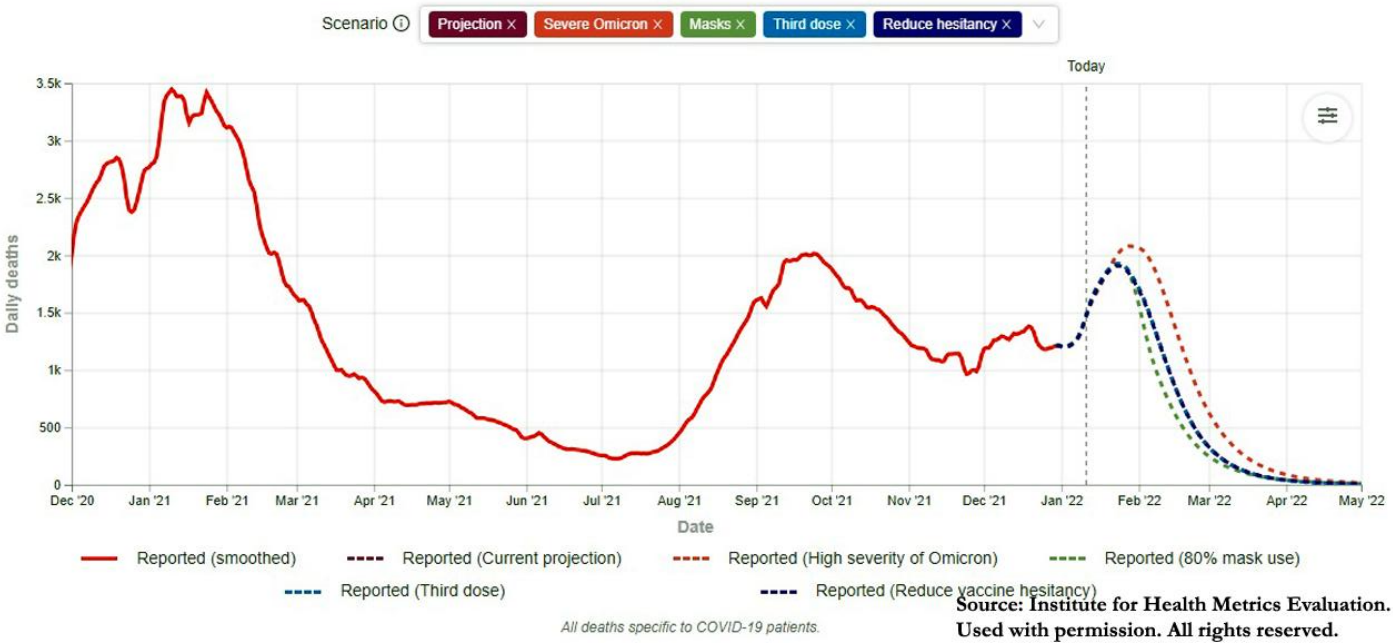
[518] Hasan et al., "The Global Case-Fatality Rate," 2179.
[519] Coronavirus Resource Center, Johns Hopkins Univ. (Jan. 4, 2022), at https://coronavirus.jhu.edu/data/mortality.
[520] WHO, "Estimating Mortality from COVID-19," 2.
[521] Luo et al., "Infection Fatality Ratio and Case Fatality Ratio of COVID-19," 43.
[522] On 1/26 the John Hopkins Univ. tracker listed 15,895 deaths for the past week.
[523] CDC, "COVID-19 Forecasts: Deaths" (updated Jan. 26, 2022).



Source: Institute for Health Metrics Evaluation. Used with permission. All rights reserved.

A low CFR like 1.5% may be reassuring, but it should not produce the in-caution too often seen. The sheer number of those confirmed with COVID-19 in a pandemic make 1.5% CFR translate into hundreds of thousands dead. And calling COVID-19 'no worse than season flu' is dangerously irresponsible. The evidence resoundingly says otherwise.

In one study, a nationwide cohort study in France of 89,530 patients, COVID-19 was compared with seasonal influenza. Data was collected for COVID-19 for a 2 month period (March–April, 2020) and compared to data collected for pre-pandemic seasonal influenza for a 3 month period (December, 2018–February, 2019). Almost twice as many were hospitalized for COVID-19 in its shorter period of time, with a significantly higher proportion admitted to the Intensive Care Unit (ICU), and in-hospital mortality was nearly three times higher than for flu.[524]

Another study reported deaths due to various causes in the United States for the period 2020–2021.[525] Here is a tabular summary of some data:

Deaths from COVID-19	% of all deaths (6,620,936)	Deaths from Influenza*	% of all deaths (6,620,936)
814,806	12.3%	9,579	0.1%

*Deaths *include* influenza + COVID-19 and/or pneumonia listed as cause of death.

It doesn't take a math wizard to see that COVID-19 is far deadlier than influenza alone, even when influenza deaths incorporate comorbidity with COVID-19 and pneumonia. At the same time, it is a sobering reality that because severe COVID-19 is—as its name declares— 'severe acute *respiratory* disease,' it is commonplace to find pneumonia and influenza as comorbidities. In fact, this pair constitutes the most common comorbidities in fatal cases of COVID-19. Of 814,806 deaths in the U.S. from COVID-19, 396,382 (48.6%)—nearly half—featured these as conditions contributing to death.[526]

In early 2022, concerns were raised about so-called 'Flurona'—a name derived from a case of a person having both COVID-19 and seasonal flu.[527] Such instances are, of course, rightly concerning. A study of cases in California—a hotspot for use of this label—found between September, 2020–April, 2021, that 23% of those with a positive influenza test result also had a matching SARS-CoV-2 result.[528] In an active flu season while the pandemic persists this constitutes another worrying concern.

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[524] Piroth et al., "Comparison."

[525] CDC (National Center for Health Statistics), "Weekly Updates by Select Demographic and Geographic Chracteristics," Table 1.

[526] CDC (National Center for Health Statistics), "Weekly Updates by Select Demographic and Geographic Chracteristics," Table 3.

[527] Scribner, "Experts Have Discovered the New 'Flurona'."

[528] Rizzo et al., "Influenza and SARS-CoV-2 Co-Infections," 2925.

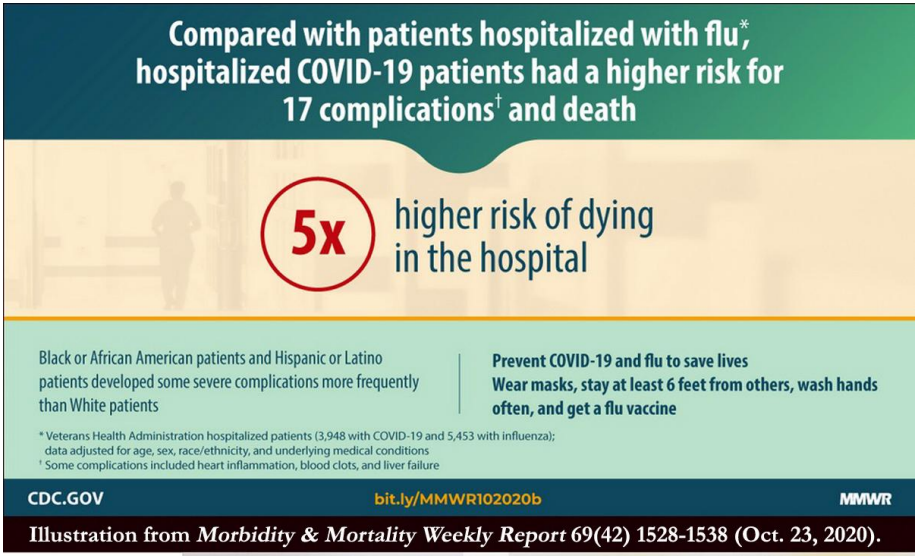

Illustration from *Morbidity & Mortality Weekly Report* 69(42) 1528-1538 (Oct. 23, 2020).

COVID-19 vs. the Common Cold

Early in 2022 another ridiculous claim began to be heard in anti-vax circles—the Omicron variant is like the common cold and no more dangerous. We saw earlier that some human coronaviruses (229E; NL63; HKU1; OC43) cause mild upper respiratory infections. The Omicron variant *might* be pointing to SARS-CoV-2 mutating in that direction, but Omicron is *not* the common cold. Natural anxiety makes it tempting to see hope around every corner after two years of a pandemic, but good science creates realistic expectations rather than emotionally-fueled fantasies.

Anti-vax claims are often rooted in a kernel of fact distorted and exaggerated for propagandistic purposes. That may play in here because of some intriguing research published in early 2022. Interestingly, having a cold derived from another coronavirus (which account for 10–15% of colds) prior to exposure to Omicron may provide some protection from infection. This might happen because of *cross-reactive immune responses*—after infection by a milder coronavirus the body's T-cells (secreting IL-2) 'remember' what the spike protein of a coronavirus looks like. Upon exposure to SARS-CoV-2 the T-cells, expert at targeting the more conserved regions of the virus (which means they are less likely to be fooled by mutations), go to work and—perhaps—foil infection.[529] But as one microbiologist noted, "It seems unlikely that everyone who has died or had a more serious infection, has never had a cold caused by a coronavirus."[530] So while the research is intriguing it ought not to persuade people that having a cold offers them immunity for some period of time or that Omicron is only as serious as a cold. Either conclusion would be foolish.

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[529] Kundu et al., "Cross-Reactive Memory T Cells." See, especially, p. 3.
[530] Cellular microbiologist Simon Clarke quoted in Schuster-Bruce, "Scientists Say."

'Long COVID' (Post-Acute COVID Syndrome (PACS))

COVID-19 soon became associated with a distressing condition known as *long COVID*—more formally known as either *post-COVID-19 manifestations*, or as *post-acute COVID syndrome (*or *sequelae)* (PACS)—in which patients continue to be plagued by symptoms for more than 4 weeks (29+ days). The condition can be subdivided by duration into two groups:

- *sub-acute PACS*—duration of less than 12 weeks (84 days), and
- *PACS*—duration greater than 12 weeks (84 days).[531]

People afflicted with this condition are sometimes referred to as *long haulers*.

One study, of 287 recovered COVID-19 patients (184 Female; 20–60 yrs. old), reported about 90% of recovered patients experienced some post-COVID-19 manifestation. The most commonly reported symptoms (in descending order of frequency):

Condition		Condition	
fatigue	72.8%	chest pain	28.9%
anxiety	38%	dementia	28.6%
joint pain	31.4%	depression	28.6%
continuous headache	28.9%	dyspnea	28.2%[532]

Another study, in Italy of 143 recovered patients, found 87.4% reported at least one post-COVID symptom, with—in descending frequency—fatigue, dyspnea, joint pain, chest pain, and cough the most commonly reported.[533]

A systematic review of 57 studies, covering 250,351 survivors of COVID-19 (56% male; 79% of all patients having been hospitalized), found more than half experienced PACS at 6 months after recovery. This study examined 38 symptoms, which were grouped into one or another of three categories:

- *organ systems* (neurologic, mental health, respiratory, cardiovascular, digestive, dermatologic, and ear, nose, and throat);
- *general and constitutional symptoms* (fatigue or muscle weakness, fever, flu-like symptoms, general pain, joint pain, muscle pain, weight loss); and,
- *functional mobility* (impairment in general functioning, mobility decline, reduced exercise tolerance).

Difficulty concentrating and memory impairment were the most common neurologic symptoms reported in various studies; anxiety or depression, and sleep disorders were the most common mental health issues. Chest pains and heart

[531] Cervia et al., "Immunoglobulin Signature Predicts Risk," 1.
[532] Kamal et al., "Assessment and Characterization of Post-COVID-19 Manifestations," Table 2. Summary by organ system: Nalbandian et al., "Post-Acute COVID-19 Syndrome," 603 (Box 1).
[533] Carfi et al., "Persistent Symptoms in Patients After Acute COVID-19." See 605 (Figure).

palpitations were common cardiac manifestation. Dyspnea and cough were not uncommon respiratory issues. Gastrointestinal issues included decreased appetite, diarrhea, and vomiting. Some impairment in general functioning or mobility, including a reduced tolerance for exercise, was reported by various studies. Finally, a number of the reviewed studies noted 'persisting symptoms'—fatigue or muscle weakness, flu-like symptoms, general pain, muscle pain, persistent fever, and weight loss.[534] This recounting by no means covers all the symptoms reported in the various studies reviewed. The general points of such a review are clear enough: some form of long COVID is not uncommon, may take any number of forms, and may be accounted for by some combination of viral effects and the toll on mental health exacted by the process of illness and recovery.

Long COVID has been found even in young, low risk patients. For example, a late 2020 report in England found that almost 70% of 201 patients in this group continued to experience impairments in one or more organs four months after initial symptoms.[535] A later meta-analysis of 22 studies, involving 23,141 children and young people, found 101 symptoms reported as 'persistent' post-COVID. But focusing on the pooled risk difference in symptom prevalence between COVID-19 cases and controls researchers reported small but significant differences in only five matters (cognitive difficulties, headache, loss of smell, sore throat and sore eyes).[536]

In early 2022, a study reported discovery of a distinct *immunoglobulin* (Ig) *'signature'*—a specific pattern—associated with long COVID. The research involved 175 COVID-19 confirmed cases (by RT-PCR testing) and 40 healthy individuals ('controls'). A pattern became obvious when comparing and contrasting Ig types and levels in different COVID-19 cases against those levels found in the healthy controls. The relative differences in total serum concentrations can be portrayed like this ('indifferent' meaning not significantly different):

	Mild COVID-19	**Severe COVID-19**	**PACS COVID-19**
IgG_1	indifferent	indifferent	indifferent
IgG_3	higher	higher	***lower***
IgM	indifferent	lower	lower

No significant differences were found in IgA, IgG_2, or IgG_4. The most evident difference—highlighted above in boldface italics—was in IgG_3 being *lower* among patients who developed long COVID. Interestingly, persons with low levels of either IgM or IgG_3 were at increased risk of PACS while those high in both were at decreased risk. The researchers also found that this Ig signature was part of a larger pattern predictive of developing PACS, one associated also with increased age, history of asthma bronchiale, and other factors.[537]

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[534] Groff et al., "Short-Term and Long-Term Rates of Postacute Sequelae of SARS-CoV-2."
[535] Iacobucci, "Long Covid."
[536] Behnood et al., "Persistent Symptoms Following SARS-CoV-2 Infection," 1–2.
[537] Cervia et al., "Immunoglobulin Signature Predicts Risk." See especially p. 3, 5–6.

Multisystem Inflammatory Syndrome in Children (MIS-C)

Another very concerning special condition found with COVID-19 patients has been *multisystem inflammatory syndrome in children* (MIS-C), so-named by the U.S. Centers for Disease Control and Prevention (CDC) on May 14, 2020.[538] It also is sometimes referred to as *pediatric inflammatory multisystem syndrome* (PIMS), and has been grouped with similar conditions under the broader heading of *pediatric hyperinflammatory conditions* (PHIC).[539]

Although the CDC considers the uppermost age limit for diagnosis to be age 21, the World Health Organization (WHO) specifies ≤19 years old. Diagnostic criteria are:

- *fever* (100.4F or higher/38C or higher) for at least 24 hours;
- *elevated inflammatory markers* (e.g., elevated C-reactive protein (CRP), or lactic acid dehydrogenase (LDH)), as measured by laboratory testing;
- *hospitalization with severe illness involving multiple organ dysfunction* (cardiac, renal, respiratory, hematologic, gastrointestinal, dermatologic or neurological);
- *current or recent SARS-CoV-2 infection* (as shown by antigen, RT-PCR, or serology test), or *exposure to COVID-19* (within 4 weeks of symptom onset); and,
- *exclusion of other plausible diagnoses*.[540]

WHO's criteria for COVID-19-associated MIS-C require meeting at least two of five criteria, which include signs involving skin, mucous membranes or eyes (e.g., rash or pink eye without pus); low blood pressure (*hypotension*) or shock; heart issues (e.g., myocardial dysfunction); bleeding issues (*coagulopathy*); and acute gastrointestinal problems (diarrhea, vomiting, or abdominal pain).[541]

Among pediatric and adolescent hospitalizations associated with COVID-19, a systematic review and meta-analysis of 14 studies, with 2,202 patients, found an estimated pooled prevalence of PHICs to be more than one-quarter (29%) of patients.[542]

A systematic review of 16 studies published on of MIS-C associated with COVID-19, covering 655 patients (55% male), found fever to be the most predominant symptom at hospital admission. But gastrointestinal manifestations—abdominal pain, diarrhea, nausea, vomiting—were also quite common (70%). Cardiovascular problems were also frequently reported (51%). Central Nervous System (CNS) issues were observed for a little more than a fifth (22%) of pa-

[538] Kaushik et al., "A Systematic Review," e340.
[539] Sharma and Bhaskar, "Prevalence of Paediatric Hyperinflammatory Condtions," 101.
[540] CDC, "Information or Healthcare Providers about Multisystem Inflammatory Syndrome in Children (MIS-C)."
[541] Jiang et al., "COVID-19 and Multisystem Inflammatory Syndrome," e277 (Table 1).
[542] Sharma and Bhaskar, "Prevalence of Paediatric Hyperinflammatory Condtions," 106.

tients. Circulatory shock (26%) was also an issue.[543] Similarly, in a pooled meta-analysis, nonspecified gastrointestinal complaints emerged as the most frequently reported clinical feature (88%), with vomiting (68%), diarrhea (48%), and abdominal pain (19%) also commonly reported. Neurological symptoms emerged more than a third of the time (36%). Inflammation of the heart muscle (myocarditis) was found in just more than half of cases (51%), and laboratory/radiological features included cardiac dysfunction in two-thirds (67%) of the cases. Shock and multiple organ failure were real concerns.[544]

Although similar in presentation to Kawasaki disease, there are important differences, such as Kawasaki occurs principally in Asian children age five or under, while MIS-C are typically over age 7 and show broader ethnic origins.[545] However, certain minorities—Black and Hispanic—may be at greater risk.[546] In MIS-C the median age has been reported to be 7.3–10 years old, with more than half (59%) male.[547] Any link between Kawasaki disease and coronaviruses remains uncertain, with various studies affirming or denying a possible connection to HCoV-NL63 (with the weight of the evidence denying a link).[548]

The review mentioned earlier, covering 655 patients, reported the median duration from symptom onset to hospitalization was 4 days (range 3–6 days). [549] Guidelines for treatment vary from country to country; there were no unified guidelines present at the end of 2021.[550] The most common treatment in hospitalized cases (76%) was *intravenous immunoglobulin* (IVIg)—a procedure in which antibodies (immunoglobulins) are taken from the blood plasma of healthy donors and then administered through the patient's veins (intravenous); the dosage depends on the patient's weight. Other common treatments, both used in about half of cases, include *steroids* (52%) or *vasoactive medications* (53%)—commonly used to treat shock they affect blood pressure and heart rate.[551]

Fortunately, the incidence of MIS-C is low and outcomes are generally favorable. In the United States, incidence of MIS-C was about 2 per 100,000 persons (of the same age group). While among those admitted to a hospital the admission rate to the intensive care unit (ICU) can be high (72%),[552] the mortality rate has hovered at around 2%.[553]

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[543] Kaushik et al., "A Systematic Review," e341.
[544] Jiang et al., "COVID-19 and Multisystem Inflammatory Syndrome," e282 (Figure 3) and e277.
[545] Nalbandian et al., "Post-Acute COVID-19 Syndrome," 610.
[546] Abrams et al., "Multisystem Inflammatory Syndrome in Children," 52. Also see Rafferty et al., "Multisystem Inflammatory Syndrome in Children," 485.
[547] Abrams et al., "Multisystem Inflammatory Syndrome in Children," 48.
[548] Jiang et al., "COVID-19 and Multisystem Inflammatory Syndrome," e278.
[549] Kaushik et al., "A Systematic Review," e341.
[550] Algarni et al., "Clinical Practice Guidelines in Mutlisystem Inflammatory Syndrome (MIS-C)."
[551] Abrams et al., "Multisystem Inflammatory Syndrome in Children," 48. Also see Rafferty et al., "Multisystem Inflammatory Syndrome in Children," 490
[552] Tang, "Multisystem Inflammatory Syndrome in Children."
[553] Rafferty et al., "Multisystem Inflammatory Syndrome in Children," 485, 491. Cf. 1.7% in Kaushik et al., "A Systematic Review," e343.

Testing for COVID-19

In the anxiety and fear occasioned among many by COVID-19 there often has been on one side a misplaced confidence or on the other a misplaced distrust of testing for the virus and disease. As a 2020 review of the matter aptly and accurately put it, "No test gives a 100% accurate result."[554] This is as true in medical testing as in educational and psychological testing. COVID-19 testing aims at achieving two things:

- High *sensitivity*—a true positive rate of infection. This means that when a positive test result is obtained indicating an infection that result is true. Sensitivity reflects the proportion of patients with the disease (100%) who also have a positive test (e.g., 98%).
- High *specificity*—a true negative rate. This means that when a negative test result is obtained indicating no infection that result is true. Specificity reflects the proportion of patients without the disease (100%) who also have a negative test (e.g., 98%).[555]

Sensitivity and specificity are considered together. In both sensitivity and specificity the result is measured against the total population of those who share a particular condition—either all those who have the disease, or all those who do not. When considered together a better measure of the test can be achieved.

There are tests both to determine if one is presently infected and if one has previously been infected. Here our focus is on the detection of present infection. (The matter of testing for previous infection is discussed in the next chapter while considering questions of immunity.)

There are different kinds of *diagnostic tests* approved in the United States for the detection of SARS-CoV-2 infection.[556] These include:

- *antigen tests* look for specific proteins made by SARS-CoV-2;
- *molecular tests* look for the virus itself through its genetic material; and,
- *serology tests* look at antibody levels following infection.

Rapid result antigen tests and molecular tests both have high sensitivity—they are excellent in accurately reporting true positives. But they vary more in reporting specificity. Molecular tests are nearly perfect in reporting true negatives, while rapid antigen tests gravitate toward the 80–90% range in specificity.[557] This increased possibility of a false negative is why, during the Omicron wave,

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[554] Watson, Whiting, and Brush, "Intreting a COVID-19 Test Result," 1.
[555] See Watson, Whiting, and Brush, "Intreting a COVID-19 Test Result," 2.
[556] For an overview, see the U.S. Food and Drug Administration (FDA) webpage titled "COVID-19 Frequently Asked Questions" (https://www.fda.gov/emergency-preparedness-and-response/coronavirus-disease-2019-covid-19/covid-19-frequently-asked-questions).
[557] Heilweil, "Rapid Tests, Omicron, and You."

people were recommended to retest if they first had a negative antigen test result.

Antigen Tests

Antigen tests look for the 'antigen,' the agent that provokes the immune system response. Such tests have a strong track record in detecting active infections. The most well-known antigen part of SARS-CoV-2 is its spike (S) protein. However, the nucleocapsid (N) protein is a common choice for antigen tests because it is highly abundant in virions and infected cells.[558] These are done by a nasal swab. Though less accurate than molecular tests they typically return results more quickly.

With the Omicron variant that emerged at the end of 2021, new concerns were raised about the usefulness of antigen tests. A preprint study, examining a 30 person high-risk occupational cohort who had daily testing during December, 2021, reported that *most Omicron cases were infectious for several days before being detected by antigen tests*. The researchers speculated that antigen tests might be less useful for Omicron compared to previous variants because of the shorter time from virus exposure to infectiousness and because lower infectious doses still suffice for viral transmission.[559]

Molecular Tests

Molecular tests look for the 'molecule.' Both the polymerase chain reaction test (PCR) and the reverse transcription polymerase chain reaction (RT-PCR) test are molecular tests. The latter, as the name suggests, is a type of the former.

RT-PCR Test

The *reverse transcription polymerase chain reaction* (RT-PCR) *test* has the ability to detect RNA. COVID-19 is typically diagnosed following the use of a RT-PCR test. Toby Lowe, associate director for the FDA's In Vitro Diagnostics Program (involved in assessing and approving COVID-19 tests), describes molecular tests this way: "So molecular tests work by looking for genetic material from the virus, and they are doing that by using a sample from a patient's nose, such as a nasal swab. This sample is then usually sent to a laboratory to be analyzed. And for some tests, the sample can be analyzed at the point-of-care, such as at the doctor's office."[560] Results, depending on the particular test, might be returned within minutes or require days.

How accurate are they? Remember, as we discussed at the start of this section, no test can guarantee being perfectly right all of the time. With respect to tests for COVID-19, the review cited earlier found *false negative rates* (FNR—i.e., a

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[558] Ye, Lu, and Corbett, "Structural Basis for SARS-CoV-2 Nucleocapsid Protein Recognition," 2.
[559] Adamson et al., "Discordant SARS-CoV-2 PCR and Rapid Antigen Test Results." See esp. p. 3.
[560] Lowe is quoted in an interview with FDA Deputy Commissioner for Medical and Scientific Affairs, Arnand Shah; see FDA, "FDA Insight: All About COVID-19 Testing."

test result saying no virus infection is present when in fact it is) of between 2–29% based on initially negative test results that upon retesting were positive.[561] Now clearly there is a significant difference between 2% false negatives and 29% false negatives. But rather than see the glass as half empty, consider this: a 29% false negative—the extreme end of that possibility—still represents a sensitivity of 71% for the test, which is far better than a guess. At the other extreme, a false negative of 2% means the test's sensitivity is 98%—almost always right.

No one likes to see such variance in sensitivity, but it can be explained. A number of factors can affect the sensitivity of a RT-PCR test. They include:

- where in the body the sample was taken;
- the quality of the sample;
- which gene targets were used;
- whether multiple gene tests in combination were used;
- the stage of the illness when the sample was taken;
- the degree of viral multiplication or clearance in the person; and
- whether the test results were determined in the laboratory or not.[562]

RT-PCR tests also fall short of 100% in sensitivity—true positives (i.e., the test says an infection is present and it really is). Some research has indicated sensitivity of 83.3% (compared to 97.2% using CT scans).[563] Thus, while RT-PCR tests are sometimes referred to as 'the gold standard' in testing for COVID-19, some researchers have called for repeated testing when COVID-19 is suspected but a RT-PCR test comes back negative,[564] or supplementing such tests with others, like a computerized tomography (CT) scan.[565]

Nucleic Acid Amplification Test (NAAT)

Another kind of molecular test is the *nucleic acid amplification test* (NAAT). As the name indicates, the test works by making many copies (the 'amplification') of viral genetic material (the 'nucleic acids') obtained from a patient sample. Such tests collect material from either the upper or lower respiratory tract. A NAAT with a RT-PCR assay to detect viral material in the upper respiratory tract is common.

In upper respiratory specimens, those that are nasopharyngeal (i.e., area of throat behind the nose), nasal, or saliva specimens show somewhat better results than those obtained by oropharyngeal swab (i.e., from the throat at the back of the mouth). Nasopharyngeal specimens, obtained by swabs, are widely

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[561] Watson, Whiting, and Brush, "Intreting a COVID-19 Test Result," 1. Research reported by Arevalo-Rodriguez et al., "False Negative Results of Initial RT-PCR Assays,"11, found FNR ranging from 0.018 to 0.58 (Median of 0.11) across 34 studies.

[562] Watson, Whiting, and Brush, "Intreting a COVID-19 Test Result," 1.

[563] Long et al., "Diagnosis of the Coronavirus Disease (COVID-19): rRT-PCR or CT?" 4.

[564] Arevalo-Rodriguez et al., "False Negative Results of Initial RT-PCR Assays,"14.

[565] Marateb et al., "Automatic Classification between COVID-19 and Non-COVID-19," 10. Cf. Yang et al. "Diagnostic Classification."

regarded as the 'gold standard' for sampling. Because the procedure for obtaining such samples is uncomfortable and somewhat complicated by other issues, alternatives have been developed. A systematic review and meta-analysis of 23 studies (from 5,577 sampled), covering different regions of the world (Asia, Eastern Mediterranean, Europe, North America) and 7,973 individuals, examined 16,762 respiratory samples (using standardized RT-PCR procedures). Since all the studies had used nasopharyngeal samples these provided a base against which other sampling done could be compared. Thus, using nasopharyngeal swab as a reference standard the alternatives looked like this:

Test Sample (by swab)	**Sensitivity** (True Positives)	**Specificity** (True Negatives)
Saliva	85%	99%
Nasal	86%	99%
Throat	68%	97%
Nasal & Throat (pooled)	97%	99%

As the above table shows, the researchers concluded that the best alternative to nasopharyngeal swab sample-collecting was pooled nasal and throat swabs.[566]

With the persistence of the pandemic, *testing has improved*. But the bottom line remains the same as it was early in the pandemic. While positive RT-PCR test results are useful for decisions such as seeking treatment and/or undergoing isolation for the duration of illness, a negative RT-PCR test should not be assumed definitive; a person whose suspicions prompted them to get tested is best served still observing sensible precautions.

Serology Tests

Serology tests, rather than being used to detect and confirm infection like the antigen and molecular tests, measure the immune system response to infection. In a process known as *seroconversion* antibodies produced in response to infection begin to show up in blood serum. So serology tests measure the antibody levels found in blood. Hence they require a blood sample. Typically such samples are drawn by a needle in a vein, just as other diagnostic blood samples (e.g., for cholesterol) are drawn. This kind of test is normally done 20 or more days after symptom onset. They generally follow cases of COVID-19 confirmed by a RT-PCR test (discussed above).

With COVID-19 *serosurveys* have been employed to determine how many people in a given population have been infected by SARS-CoV-2.[567] A serosur-

[566] Tsang et al., "Diagnostic Performance of Different Sampling Approaches." The authors also report *positive predictive values* (PPVs), the probability of actually having COVID-19 after getting a positive result, and *negative predictive values* (NPVs), the probability of not having the disease after receiving a negative result.

vey tests blood serum drawn from a representative sample of the community being studied (the 'target' population). A s*eroprevalence survey* measures the proportion of a target group that has antibodies to the virus. Samples that are negative for viral antibody suggest susceptibility to the virus.

There was great hope early on that serosurveys would be very useful with COVID-19. In fact, initial serosurveys identified antibodies in nearly 100% of persons with RT-PCR–confirmed SARS-CoV-2 infection. But success stories have proven somewhat illusory. Subsequent research has found seroconversion rates are surprisingly variable. For example, in one study 72 patients who had a previous positive RT-PCR test, but were symptom free for at least three weeks, received a serology test. More than a third of these proved to be serologic non-responders (i.e., people who showed no sign of previous infection)—and this result mirrored other studies finding seronegative results in anywhere from 5% to 85% of their subjects. In their own study the researchers found that patients with low SARS-CoV-2 viral loads in their respiratory tract were less likely to mount a systemic antibody response.[568]

Another study found that RT-PCR test results upon first clinical presentation of COVID-19 differed from subsequent serology tests in about 1-in-5 patients. More often the discrepancy was between an initial negative RT-PCR (i.e., the test indicated no infection) and subsequent positive serology (i.e., antibodies showing previous infection). Such patients were associated with cases of COVID-19 with fewer symptoms than was observed in patients with positive results for both RT-PCR and serology tests. In cases were a positive RT-PCR result was not also accompanied by a positive serology test, patients tended to display a greater number of symptoms and a longer course of disease.[569]

Testing vs. SARS-CoV-2 Variants

With each new variant of SARS-CoV-2 questions are raised concerning the effect that will be seen on COVID-19 tests. For example, the U.S. Food and Drug Administration (FDA) reported in late December, 2021, that *antigen tests can detect Omicron but may have reduced sensitivity compared to earlier variants*. (See the material above on antigen tests for more on this point.) Because of this, the FDA recommends that upon a negative test result a person with symptoms consistent with COVID-19 should follow-up with a molecular test (RT-PCR).

At the same time, the FDA noted that the approved Tide Laboratories DTPM COVID-19 RT-PCR Test was highly successful with both the Delta and Omicron variants. This followed modification of the test. The FDA also observed that molecular tests designed to detect multiple genetic targets may retain usefulness in detecting Omicron.[570]

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[567] Murhekar and Clapham, "COVID-19 Serosurveys."
[568] Liu et al., "Predictors of Nonseroconversion."
[569] Baron et al., "Frequency of Serological Non-Responders," 2136.
[570] FDA, "SARS-CoV-2 Viral Mutations: Impact on COVID-19 Tests."

COVID-19 caused by SARS-CoV-2 is an enemy to which Americans have responded in varying ways. But all of them resolve down to one of two basic alternatives: taking one's chances that either no infection will happen or if it does that it will prove of no serious consequence, *or* trying both to prevent infection and limit the likelihood of serious consequences if it does occur. To use our war metaphor, the difference is in either relying on the 'country' (i.e., one's body) to respond quickly and effectively in the event of invasion (i.e., through a natural immune response), or relying on preparing the country in advance through calling up the national guard and drafting new soldiers (i.e., preparing an immune response in advance of invasion). The latter path is taken by pro-vaccine advocates. Anti-vaxxers choose instead the former path. The consequences of selecting one or the other path are markedly different—and do not favor an anti-vaccine choice.

Chapter 5
The Human Immune System

The word 'immune' derives from the Latin *immunis*, a term signifying an 'exemption'—back then it meant exemption from public service offices. In essence it meant being 'protected' against being made to do something. The logic has been reoriented these days to refer to *protection against agents that threaten the body*. The immune system aims to protect and through its action provide ongoing protection. It is this latter idea that people emphasize today when they talk about having 'immunity' from some disease.

There are *two paths to immunity* against SARS-CoV-2:

- immunity can be gained from the human immune system responding to *natural infection* by the virus; and,
- immunity can be gained from the human immune system responding to *vaccination*.

This chapter deals with the first path. Please note that both paths involve the immune system; some people seem to imagine that vaccination is off to one side and therefore is not as 'good' as the immunity from natural infection. Whether their conclusion is correct or not we'll deal with later, but the above line of reasoning to get to such an idea is flawed.

A formal definition for immunology as understood today runs like this: "the study of the recognition of non-self entities; the activation, development, and specific defense functions of lymphocytes; the interactions of lymphocytes with other cells; and the investigation of the genes and proteins underlying these interactions."[571]

That definition contains a lot of information we need to unpack. Let's start with the idea of *non-self*. The host immune system is tasked with recognizing what belongs in the human body and what doesn't ('non-self' things). Those things that don't belong are called *antigens*, a compound word meaning 'against' (*anti-*) and 'things that cause' (*-gens*)—so things that cause actions against the body, like agents of disease. More specifically they have come to refer to the substances (such as a surface protein on a virus) that provoke an immune response. People are familiar with antigens as those things that provoke allergies.

The next three things—activation, development, and specific defense functions—are all linked to the word *lymphocytes* (literally, 'clear water' (*lympho-*) 'hollows' (*-cytes*)), that is, cells that physically look like clear water compared to, say, red blood cells. In fact, many people know lymphocytes as *white blood cells*. They look clear ('white') in contrast to the cells around them. As the next point in the definition makes plain, these lymphocytes interact with other cells in the complex coordination of the immune system.

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[571] Mak and Saunders, *The Immune Response*, 7 (italcized in the original source).

Finally, as we have been working with throughout this volume, immunology is about *genes and proteins*. These are the biological instructions (genes) and building blocks (proteins) that make the system. That aspect can't be avoided, but because we are staying at the shallow end of the pool we will stay pretty basic.

In this chapter we will first attempt to capture a general sense of how the human immune system operates. Then, next chapter, we will turn specific attention to how it contests SARS-CoV-2. Those already familiar enough with the immune system may therefore wish to press ahead to that section, though a brief review of what precedes it may still be profitable.

Overview of the Human Immune System

Although this chapter speaks of general characteristics of the human immune system we must start with recognizing individual immune systems are not equal. It is a mistake to imagine everyone's immune system works as well as everyone else's. The fundamental architecture and plan is the same, and the players are familiar ones, but variability is the name of the game. Put most simply, human immune systems vary in their performance across individuals, meaning whatever is said about how the immune system responds to SARS-CoV-2 is *generally* correct but does not guarantee that same response in every person.

Variability in the Species, Stability in the Individual

As is true of so many things, there is higher variability *between* individuals than *within* any given person. Thus from person to person we find more variability than occurs over the lifespan of a particular person. The variability among people reflects a basic characteristic of adaptation such that a population faced with a particular health risk will have some members more resistant to that threat and thus more likely to survive and pass on the immune system characteristics best fitted to that environment. In addition, for reasons still unclear, there is some variability among individuals that is influenced by sex; for example, females are more susceptible to autoimmune diseases.

Though the human population as a whole shows high variability, that is not the case generally for individuals. Evidence shows instead individually stable baseline states that are well-balanced and regulated across time. In healthy individuals such states can persist several years unless interrupted by a health crisis. Then the system responds to meet the crisis, after which it returns toward the baseline. Typically the greatest variability in a healthy individual's lifespan will be seen in infancy as new infectious agents are encountered, and in older adulthood, as immune cells are lost and other changes reduce system effectiveness. But it is important to recognize that environmental factors and life experiences also have a significant influence in whatever changes may occur across a lifetime.[572]

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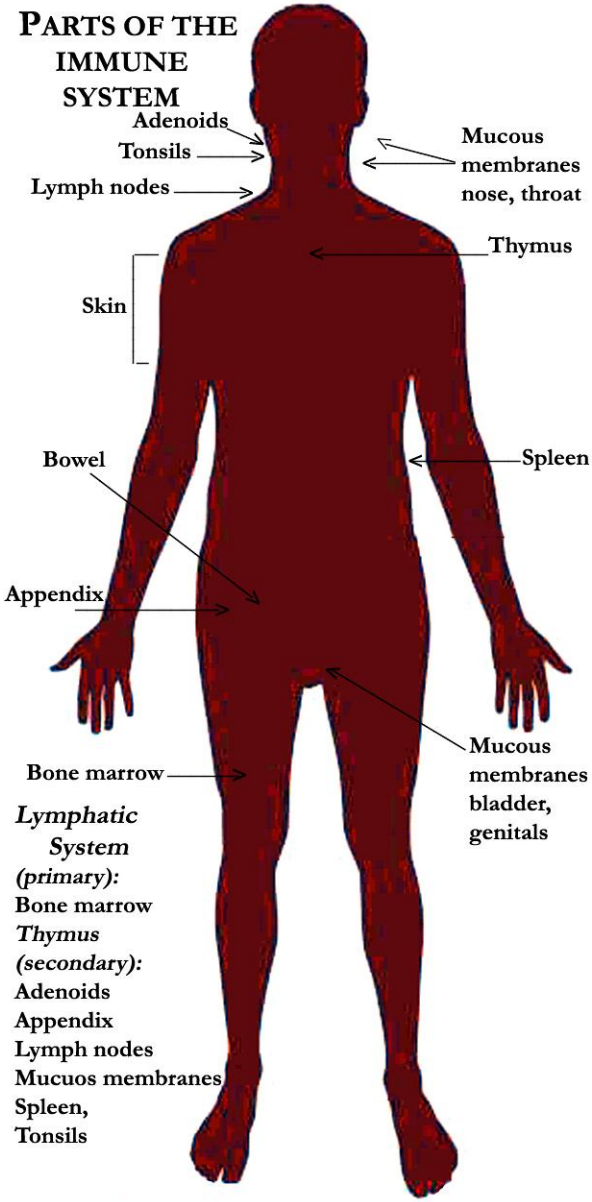
[572] This section on variability and stability is based on Brodin and Davis, "Human Immune System Variation." Specifically on sex-based variations, see Márquez et al. "Sexual-Dimorphism in Human Immune System."

Architecture of the Immune System

The architecture of the human immune system can be described in different ways. For example, it can be examined strictly in terms of the body parts that are involved (structure) and the roles they play (function). Consider, for example, the following illustration, indicating important parts of the system.



Structural Levels

Sometimes the immune system is described in terms of different *levels of structure*. Our illustration suggests this approach. Structures include:

- various *organs* (structures comprised of tissues)—for example, appendix, spleen, thymus;
- *tissues* (cells of similar structure sharing a function)—such as the adenoids, bone marrow;
- *cells* (e.g., lymphocytes);
- *extracellular fluids* (macromolecules of the *humoral system*).

The first three types of parts belong to a complex system—the *lymphatic system*, which also interacts with the *humoral system*. The adjective 'lymphatic' is derived from the Latin word *lymphaticus*, which denoted someone stricken with an anger much like an imagined nymph's wrath, thus 'gripped with madness.' But the root *lympha* is derived from an adjective meaning 'clear' and lymphatic fluid is a clear, watery substance. Both senses might at times seem to apply; the literal sense of a fluid always characterizes the system, but metaphorically one may feel like they are 'going mad' when the system is engaged in its work fighting enemies in the body. The lymphatic system is a network that, as shown in the illustration, is comprised of organs and tissues. This network transports *lymph*, a fluid bearing white blood cells (lymphocytes) to fight infection.

This way of looking at the immune system is familiar and helpful. We shall consider the parts of the lymphatic system as we go along. But there is also value in considering a developmental approach to the structure of the immune system. For our system develops, and because it does the system is often referred to as though it were two systems. It is, in fact, a two-in-one system.

Two-in-One Immune System

The immune system is built by exposure to environmental agents but already at birth we have a ready—'innate'—defense. In broad strokes the immune system includes:

- an *innate* immune system present at birth, and,
- an *adaptive* immune system developed through experience.

Our *innate* immune system—the one we are preprogrammed with—becomes paired with an *adaptive* immune system through exposure. Thus the human immune system is dual: both an innate system and an acquired one.

These two systems are also sometimes termed this way:

- a *nonspecific* immune system (the innate) that offers generalized responses to antigens and never varies or 'learns'; and,
- a *specific* immune system (the adaptive) that provides specific responses and 'learns' and 'remembers' so that those responses get better over time.

Here is the short roll call of some of the key players:

Innate *(Nonspecific)*	**Adaptive** *(Specific)*
Physical barriers (e.g., skin) **Chemical barriers** (e.g., mucus, sweat, saliva, tears) **Cells:** granulocytes (e.g., basophils, eosinophils, neutrophils), monocytes/ macrophages, dendritic cells (DC), innate lymphoid cells (ILCs), natural killer (NK)	**B-cells** (*B lymphocytes*) producing immunoglobulins (Ig), also called antibodies); and **T-cells** (*T lymphocytes*)—'helper' and 'suppressor' T-cells

The cells of the immune system are typically distinguished by which of the above they belong to, though it is possible to serve both (e.g., Natural killer T-cells (NKT) are said to bridge both). The distinction between the kinds of cells follows the logic of each system. Innate immune system cells are fixed in functional character. They use receptors to recognize common molecular patterns among infectious agents and they respond in fixed ways to each exposure to those patterns. On the other hand, adaptive immune system cells 'learn'—they 'adapt' by becoming quicker and more effective in response upon repeated exposure to the infectious agents they combat.[573] Unlike cells of the innate immune system, those of the adaptive immune system have receptors that are antigen-specific.[574]

Primary Functions of the Immune System

The architecture of the immune system requires more space to make plain than does its functions. All the myriad things the immune system does can be grouped under two broad headings:

- *immune surveillance* (or *immunosurveillance*); and,
- *protective response*.

As the name offers, 'immune surveillance' refers to the primary task of overseeing and monitoring the body. The outcome of this vigilance is the 'protective response' in which appropriate immune system cells are deployed to act.

Immune Surveillance and Immune Escape

Immune surveillance is a concept most often known in connection with cancer. The hypothesis was set out in the 20th century that the immune system functioned to prevent some cells from developing into tumors and that prevention suggested an underlying readiness beforehand. Though there has been evidence presented both for and against the idea, it has become quite influential with respect to understanding immune system functioning and is a key concept

[573] Wu and Van Kaer, "Natural Killer T Cells," 236.
[574] For more, but still brief elaboration, see Bhat and Kabelitz, "An Introduction to Immunology and Epigenetics," 2.

in thinking about cancer. More recently, some have suggested instead the idea of *immunoediting*, characterized by three phases: an eliminating of cancerous cells, a resulting state of equilibrium, and an escape phase (from surveillance) in which a detectable tumor develops.[575]

Our interest lies mostly with a notion associated with immune surveillance known as *immune escape*. We have encountered this idea in earlier chapters as we considered ways in which SARS-CoV-2 evades detection. In the constant struggle between the virus and the human bodies it invades, as the immune system works to detect it, the virus works to escape that detection. The virus does so by a process of mutation—especially in the spike (S) protein on its surface—through which its presentation to host cells is subtly altered. Scientists John Moore and Paul Offit explain: "These 'escape mutations' typically arise when the virus is put under selective pressure by antibodies that limit but do not eliminate viral replication."[576] Mutations thereby aid 'escape' from immune defense.

Mutations leading to successful 'disguise' facilitate infection and replication. Virus researchers William T. Harvey and colleagues put the importance of this phenomenon in a highly relevant context: "Given that therapeutics (vaccines and antibody-based therapies) target mainly the SARS-CoV-2 spike protein, the selection pressures that favour the emergence of new variants carrying immune escape mutations generated in chronic infections will be similar to those selecting for mutations that allow reinfections within the wider population."[577]

The Omicron variant exemplifies this mutagenic evolution of SARS-CoV-2. It has 32 mutations in the spike protein, 15 of which are in the receptor binding domain (RBD) so critical to attachment and entry into host cells (as shown in chapters 2–3). As a result Omicron has proved very successful at immune escape, significantly voiding the immunity being achieved both by natural infection and vaccination.[578] This, in turn, creates an expectation that Omicron can and will produce more breakthrough infections in the vaccinated and reinfections in the unvaccinated[579]—scenarios that existing evidence supports.

The question is then raised whether scientists should engage in a game of whack-a-mole, developing a vaccine specific to Omicron—and/or future variants.[580] Some anti-vaxxers claim that vaccines encourage virus mutations and thus make an already bad situation worse. However, existing scientific research disputes that notion and instead suggests that the vaccines actually restrict the evolutionary and antigenic pathways open to the virus when compared to what happens through natural infection in the unvaccinated.[581]

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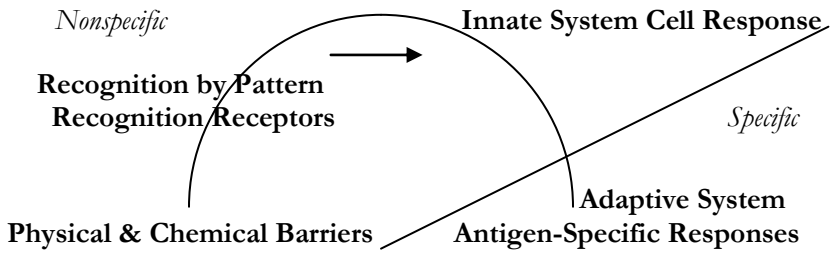
575 Ribatti, "The Concept of Immune Surveillance." On immunoediting, see Kim, "Cancer Immunoediting."
576 Moore and Offit, "SARS-CoV-2 Vaccines and the Growing Threat of Viral Variants," 821.
577 Harvey et al., "SARS-CoV-2 Variants, Spike Mutations, and Immune Escape," 422.
578 Zhang et al., "The Significant Immune Escape of Pseudotyped SARS-CoV-2 Variant."
579 Hu et al., "Increased Immune Escape of the New SARS-CoV-2 Variant of Concern."
580 See Waltz, "Does the World Need an Omicron Vaccine?" for a discussion of the issues.
581 Niesen et al., "COVID-19 Vaccines Dampen Genetic Diversity" [Preprint].

The immune system's protective response is the main thrust of our consideration in this chapter. But it presupposes the general idea of an underlying readiness that the concept of surveillance helps capture. *Host defense* is the principal nature of the immune system, its most basic function; surveillance is an outgrowth of defense.

The first line of defense is the innate immune system. As we saw in examining the architecture of the immune system, when the innate immune system is talked about it can be described at both larger (physical) and smaller (microbiological, i.e., cellular) levels. Here our attention will be where immunology most often concentrates, on the cellular level, which provides the most immediate host defense.[582] This first line of defense is inflexible. Like any fixed preprogrammed system it relies on the same set of responses to whatever is presented to it, no matter how many times it is faced by it. But because it is a varied system and operates at so many levels it provides an overall effective response. At best it can eliminate the problem; at less than best it can still serve a delaying action until the second, more sophisticated line of defense engages.

The evolved line of defense is the adaptive immune system. Kevin Schultz and Franziska Grieder capture the essence of this defense when they write, "The function of the specific immune response is aimed at an active removal of foreign antigen."[583] This highly responsive and flexible system not only can act more effectively but also develop that effectiveness further. After a first exposure to an antigen it becomes better prepared for subsequent exposures. Vaccines build on this reality by providing antigenic stimulation—a prod to the system to make it engage—but doing so with dramatically lower stakes than the health risks presented by natural infection.

These systems and their functions should not be separated too much. They are best seen as ranged along a continuum of responsiveness to antigens.[584] It might be pictured broadly like this.



As we proceed, what we examine is a matter of how the architecture and functions of the immune system involve its players in a delicate dance.

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[582] Parken and Cohen, "An Overview of the Immune System," 1777.
[583] Schultz and Greider, "Structure and Function of the Immune System," 263.
[584] Bhat and Kabelitz, "An Introduction to Immunology and Epigenetics," 5.

The Innate Immune System

The innate human system can be said to be bi-level. The two levels are:

- a macro level of inherent body barriers that non-self agents must breach; and,
- a micro level of specialized cells providing a nonspecific defense system.

While both levels matter our concentration will be on the second.

Macro Level: Body Barriers

The first level can be termed 'macro' as it refers to a large scale level relative to the much smaller level of specialized cells. All the elements of this macro level are comprised of coordinated sets of cells constituting one or another kind of barrier. Thus this level is referred to as *body barriers* against non-self agents. Such agents include infectious viruses like SARS-CoV-2. These barriers include (in alphabetical order):

- blood-brain barrier (and its like);
- chemical barriers;
- mucosal tissue; and,
- skin.

Blood-Brain Barrier, etc.

The *blood-brain barrier* (BBB) shields the brain from toxic substances that might be circulating in the blood. It is comprised principally of *brain microvascular endothelial cells* (BMVEC)—very small circulatory system vessel (*microvascular*) cells of the thin membrane lining (*endothelial*) the brain. Support is added by astrocyte and pericyte cells, and just outside the BBB, by microglia. Control of access to the brain is maintained by tight regulation of transport through physically tight junctions and use of metabolic barriers (e.g., certain enzymes).[585] Although it remains unknown whether SARS-CoV-2 can breach the human BBB, it has been shown to be able to do so in mice.[586]

To the BBB can be added two other barriers protecting the brain. The first of these is the *blood-cerebrospinal fluid barrier* (BCSFB)—or as it is also known, the *blood-CSF interface*. It is formed of the epithelial cells of the choroid plexus (tissue found in the brain ventricles). The other is the *avascular arachnoid epithelium*—a thin layer of connective tissue (*arachnoid*) covered by a thin tissue surface (*epithelium*) without blood vessels (*avascular*); it is also referred to as the *CSF-blood interface*. This last one completely encases the brain.[587]

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[585] Persidsky et al., "Blood-Brain Barrier."
[586] Rhea et al., "The S1 Protein of SARS-CoV-2 Crosses the Blood-Brain Barrier in Mice."
[587] Cippola, *The Cerebral Circulation*, 37. Also, Kardy, Noorani, and Cucullo, "A Blood-Brain Barrier Overview," 1–2.

Chemical Barriers

A variety of *chemical barriers* feature antimicrobial substances. The locations for these chemical barriers are diverse and include breast milk, mucus, saliva, and tears. A naturally occurring enzyme named *lysozyme* (or *muramidase*; chemically N-acetylmuramide glycanhydrolase) is one such antimicrobial agent, found in saliva and tears.

Another kind of chemical barrier is created by *pH levels* in the body. 'Potential hydrogen' or 'power of hydrogen' (pH) concerns how much hydrogen is in liquids—the pH level. It is expressed as a scale measure of how acidic (less than 7) or basic (greater than 7) a liquid is. Low pH—i.e., acidic levels—hinders pathogen growth. Perhaps the best known such chemical barrier is *stomach acid* (with a pH level of 1–3).

Mucosal Tissue Barrier

Mucosal tissues include the eyelids, mouth, nose, windpipe (*trachea*), lungs, stomach, and intestines—places where mucous (Latin *mucosus*, 'slimy') membranes are found. Some of these are quite visible—eyelids, mouth, nose—and commonly thought of by people as portals for invaders rather than as barriers. But while it is true that infectious agents like SARS-CoV-2 can gain entry to the body through these body parts, mucosal tissues perform a variety of complex roles including differentiating between the outside substances the body needs and those it wants to avoid—the latter facing a barrier in mucosal tissues.

The so-called *common mucosal immune system* (CMIS) stretches across the body involving many parts. The nasopharyngeal, pulmonary, and gastrointestinal tracts collectively present the largest surface area of the body exposed to the external environment. Because this system is a gate-keeper to interactions with the external world, it serves as a natural location for immune system cells. In fact, here are found more immune system cells than in all nonmucosal tissues combined.[588]

Skin Barrier

The *skin* is the body's largest organ. Together with accessories such as hair, nails, oil glands, and sweat, these form the *integumentary system* ('integumentary' being derived from the Latin *integumentum*, 'covering' or 'shield'). Skin has been called an immune system of its own—the 'skin immune system,' or more formally, *skin-associated lymphoid tissue* (SALT). Skin cells (e.g., keratinocytes, which are 90% of epidermal cells) provide a tight physical barrier against non-self items outside the body. But the skin also provides a staging area for the specialized cells of the immune system. These include both innate immune system cells (dendritic cells (DCs) and mast cells), and those of the adaptive immune system (B- and T-lymphocytes).[589]

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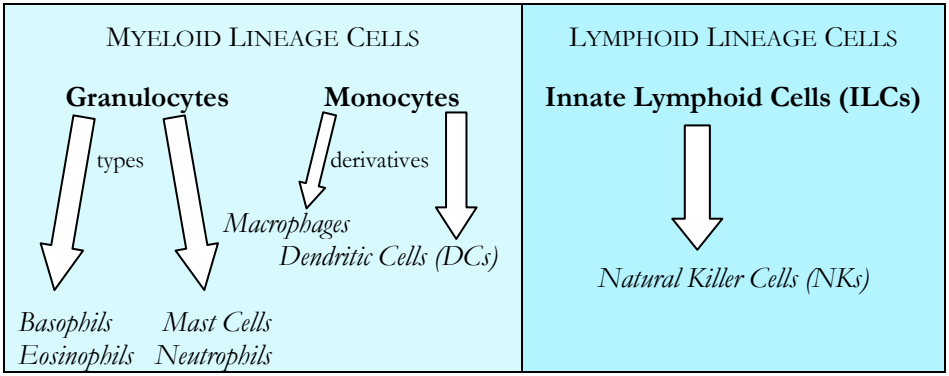
[588] Kelsall, "Mucosal Antigen Sampling."
[589] Quaresma, "Organization of the Skin Immune System."

Micro Level: Principal Cells of a Nonspecific Defense System

The second level (micro level), which we shall concentrate upon, is a *nonspecific defense system* of cells. This defense system possesses the ability to respond to a wide-range of pathogens. It both maintains constant surveillance of the body and initiates defensive actions.

The innate immune system possesses a number of specialized cells for its tasks. These include:



All of these are commonly called *nonspecific*—they are what we might term 'generalists'—able to recognize those foreign molecules common among pathogens, and then bind to them to prevent their negative effects. Traditionally they have been considered lacking any ability like that found in the adaptive immune system to learn and remember. However, mounting evidence suggests the innate immune system also may contribute to immune memory[590]—a matter briefly touched upon in what follows. Some brief general marks are placed here; specifics with respect to SARS-CoV-2 follow in the next chapter.

Granulocytes (Basophils, Eosinophils, Neutrophils, Mast Cells)

Granulocytes (sometimes called *polymorphonuclear leukocytes* (PML)), created in bone marrow, are a kind of white blood cell distinguished by *granules* (very fine particles in a cell). When granules are released the process is known as *degranulation.* The materials released are toxic to the organisms they are released against and include *defensins* (a kind of antimicrobial peptide), enzymes (e.g., *acid hydrolases*), nitric oxide, and others. Their functions are varied but a main one is engulfing a pathogen and releasing granular contents against it. Collectively granulocytes constitute the most common kind of white blood cell. They are of three types: basophils, eosinophils, and neutrophils.[591] Their life cycle movement is:

Made in BONE MARROW ⟶ released in BLOODSTREAM ⟶ Migrate to TISSUES

[590] Netea et al., "Trained Immunity: A Program of Innate Immune Memory in Health and Disease."
[591] For more, see Hägg and Kohlund, *Handbook of Granulocytes*.

Basophils are an infrequent kind of granulocyte, comprising about 0.5% of immune cells circulating in human blood. Their life span is only about 60–70 hours. Basophils function similarly to mast cells (discussed below), though with fewer and more homogenous granules. When triggered, for example by inflammation, these cells are recruited from the blood to the site where needed and release granules (minute particles) of various sizes which contain enzymatic proteins and chemicals. One of these chemicals—histamine—is well-noted for its role in allergic responses. Interestingly, while immunological memory is mostly discussed in connection with the adaptive immune system, basophils have been shown to bind intact antigens on their surface and enhance immunological memory responses.[592]

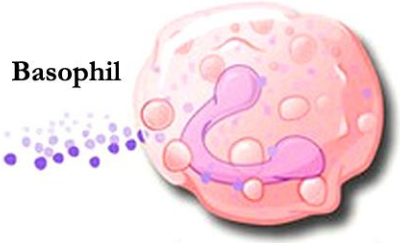


Image courtesy of National Institute of Allergy and Infectious Diseases (NIAID).

Image courtesy of National Institute of Allergy and Infectious Diseases (NIAID).

Eosinophils are a granulocyte found in many body systems. They can comprise up to 6% of the white blood cells circulating normally. Like other granulocytes they release granules to fight the body's enemies; they are perhaps best known for their role in defense against parasites and in allergic reactions. Some of their granules are proteins that are cytotoxic—to the cells (*cyto-*) thus targeted they are deadly (*-toxic*). Other granules are also 'proinflammatory,' meaning they can promote inflammation, which the immune system intends as a way to isolate and control affected sites.[593]

Neutrophils (or, *polymorphonuclear (PMN) leukocytes*) are the most abundant kind of granulocyte—about 50–70% of a normal individual's white blood cells. (In a few people genetics has intervened to create a condition known as *neutropenia*, in which there are not enough neutrophils and the individual is more susceptible to infections.) By their sheer abundance, neutrophils are like the frontline troops of an army marshaled for battle. Their typical lifespan—about a day—sees them made in bone marrow, released into blood circulation, taken up in tissues as needed, and then eliminated by macrophages (discussed below). They are characterized by three main antimicrobial functions: ingestion of the offending agent (*phagocytosis*), release of their granules (*degranulation*), and release of net-like structures (*neutrophil extracellular traps*) to engulf pathogens and then fuse their

Image courtesy of National Institute of Allergy and Infectious Diseases (NIAID).

[592] Denzel et al., "Basophils Enhance Immunological Memory Responses."
[593] For a brief general introduction see Wen and Rothenberg, "The Regulatory Function of Eosinophils."

granules to work against the pathogen. Beyond these functions they also play a role in influencing and regulating inflammation.[594]

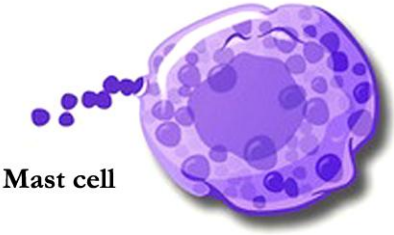


Image courtesy of the National Institute of Allergy and Infectious Diseases (NIAID).

Mast cells are granulocytes both similar and different from the three kinds just described. Like other granulocytes they are made in bone marrow, but rather than circulating in the bloodstream, mature mast cells are normally found in body tissues (epithelial and mucosal) throughout the body, but are especially notable in lung tissue, lymph nodes, liver, spleen and skin. Mast cells have been characterized as 'multi-functional master cells.' They occupy strategic locations at those places where pathogens may enter the body—which explains their location in the skin, the epithelial cells of the respiratory system, and the gastrointestinal system, among others. They hold 50–200 granules and these contain materials important in inflammation (e.g., cytokines, heparin, and histamine). As the presence of histamine might suggest, mast cells are well-noted for their role in allergic reactions. But they play many roles, including helping regulate functions of other cell types (e.g., eosinophils, dendritic cells, macrophages, B-cells and T-cells). They play important parts in eliminating bacteria and parasites. They are critical to helping maintain the relatively stable equilibrium in the body known as *homeostasis*.[595]

Monocytes (Macrophages and Dendritic Cells)

Monocytes are large white blood cells easily distinguished from granulocytes by their lack of granules. They do heavy lifting in the immune system by ingesting—and eliminating—invading microorganisms. Produced in bone marrow they circulate for a day or so in the bloodstream, then migrate into tissues where they transform into either macrophages or dendritic cells.

Image courtesy of National Institute of Allergy and Infectious Diseases (NIAID).

Macrophages acquire various more specific names in accord with whatever tissue they are found in—and they occur in tissues throughout the body. For example, macrophages in the lungs bear the label 'alveolar macrophages,' those in the CNS as 'microglia,' those in bone as 'osteoclasts,' and those in the kidneys as 'intraglomerular mesangial cells.' They also are sometimes differentiated by a classification scheme based not on location in the body but by activation and function. In this way of looking at them there are those that are said to be activated in either a 'classical' fashion (M1-type) or an 'alternative' one (M2-

[594] Rosales, "Neutrophil," 1–3.
[595] Krystel-Whittemore, Dileepan, and Wood, "Mast Cell: A Multi-Functional Master Cell."

type). Exactly how this activation takes place is less important to us here than that such activation differences lead to differences in function. M1-type phages are important in the body's defense against microbial organisms while M2-type macrophages are vital in immune system regulation and are involved in healing wounds. These functional differences are brilliantly caught in a journal article's title: "M1 Means Kill; M2 Means Heal."[596]

Dendritic cells (DCs) are those monocytes that develop a tree-like appearance with branches. They occupy a key place along the immune system continuum because, while belonging to the innate immune system they initiate adaptive immune responses. They are often referred to as a kind of *professional antigen presenting cell* (APC), which means they possess a specialized ability ('professional') to present an antigen to a T-cell. But they also can initiate innate immune responses. While immature, DCs are situated in pathways representing pathogen entry routes and via uptake of various products perform a kind of monitoring or surveillance of their environments. Matured DCs are able to migrate to T-cell areas (or migrate from non-lymphoid to lymphoid tissues—those that support immune responses).[597]

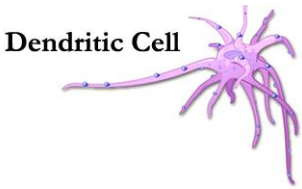


Image courtesy of National Institute of Allergy and Infectious Diseases (NIAID).

Natural Killer Cells

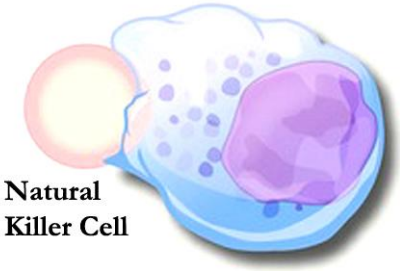


Image courtesy of National Institute of Allergy and Infectious Diseases (NIAID).

Natural Killer (NK) cells are known as 'rapid responders'—part of the first line of defense. Circulating in blood they quickly move to infected tissues and destroy body cells infected with a pathogen. They are 'proinflammatory' in that they produce cytokines. But they can also release chemokines, proteins well known for encouraging the migration of cells, especially leukocytes (white blood cells).

Traditionally NK cells have been assigned to the innate immune system. However, in recent years study of these cells has led to a more complex view of them. Other cells of lymphoid lineage—such as B-cells and T-cells—are seen as part of the adaptive immune system. NK cells have been instead termed 'innate lymphoid cells' (ILCs). Yet they appear to be involved in ways that engage other cells of both the innate (neutrophils, dendritic cells, macrophages) and adaptive (B- and T-cells) immune systems. They also exhibit evidence of some immunological memory.[598]

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[596] Ley, "M1 Means Kill; M2 Means Heal."
[597] Granucci et al., "The Immune Response is Initiated by Dendritic Cells."
[598] Vivier et al., "Innate or Adaptive Immunity? The Example of Natural Killer Cells."

A Special Note on 'White Blood Cells'

It might have been noticed in the illustration delineating the specialized cells of the innate immune system that no use of the label 'white blood cells' occurs. That is because this popular term is a bit too general to be especially helpful. A variety of kinds of white cells are part of the human immune system. They include:

- Granulocytes (basophils, eosinophils, neutrophils, mast cells);
- Monocytes (which may transform into macrophages or dendritic cells); and,
- Lymphocytes (natural killer cells, B-cells, T-cells).

But the last named—lymphocytes—largely belong to the adaptive immune system. As noted earlier, those cells of the lymphoid lineage that belong to the innate immune system—natural killer cells—are called innate lymphoid cells (ILCs) to distinguish them from the lymphocytes of the adaptive immune system.

Proteins

In discussing cells of the innate immune system there has been reference to various proteins, such as those in granules. Proteins, recall, are large molecules built from strings of amino acids and are the workhorses of cell business. There are many proteins involved in innate immune system work. Here we will list only a few with an eye on some of the better known.

- Granulocyte proteins (30–40 types of granule proteins)—*cytokines* and *chemokines*, *defensin* (DEF), *elastase* (ELA2), *lactotransferin* (LTF), *myeloperoxidase* (MPO).
- Monocyte/Macrophage proteins—classically activated macrophages (M1 type) release *inflammatory cytokines*; regulatory macrophages (R-Mϕ) release *anti-inflammatory cytokines* (*interleukin 10* (IL-10)); alternatively activated macrophages (M2 type) release *chitinase* and *chitinase-like* proteins.
- Natural Killer (NK) cell proteins—cytotoxic proteins like *perforin* and *granzyme B*, as well as *cytokines* (e.g., *interferon gamma* (IFN-γ), IL-10, and *tumor necrosis factor alpha* (TNF- α)), and growth factor proteins (for white blood cells—*Granulocyte-macrophage colony-stimulating factor* (GM-CSF) and for granulocytes and stem cells— *Granulocyte colony-stimulating factor* (G-CSF)).

Mindful of not venturing into the deeper end of the pool, our remarks on proteins will focus on how the immune system fights danger through recognizing it, alerting the system, and acting in defense. However, even a casual perusal of the above list reveals one kind of protein that appears with regularity—cytokines. It is important enough to warrant special attention right now.

Cytokines are 'cells' (*cyto-*) that 'change' (*-kine*) the behavior or status of other cells. The Greek κίνησις (*kinēsis*) refers to the kind of 'motion' or 'movement' that results in change. The name fits as cytokines label a large group—more than 100 identified just in the last decade or so[599]—of rather loosely related substances that are united in this broad sense of functioning to produce change through intercellular signaling. They often work in coordinated fashion, but they can also be *antagonistic*—i.e., one cytokine may inhibit another.

Commonly these types of cytokines are distinguished:

- *chemokines*—important for directing migration of white blood cells to where they are needed (although they do other things, too);[600]
- *interferons* (IFNs)—triggered by other cytokines or by pathogens, they have affects on cell growth, inflammation, etc.;[601]
- *interleukins* (IL-1, etc.)—more than 60 have been identified, which mediate communication between cells, regulate diverse cellular processes (e.g., cell growth and differentiation), and stimulate immune responses (both innate and adaptive);[602]
- *transforming growth factors* (TGFs)—possess numerous immune functions, including inflammatory activity;[603] and,
- *tumor necrosis factor* (TNF)—multifunctional, TNF is noted as a pro-inflammatory cytokine, but plays a role in many cellular matters.[604]

Cytokines are not just produced by cells of the immune system, though that is our focus here. Among cells of the innate immune system they are especially associated with macrophages and mast cells, while among cells of the adaptive immune system they are associated with both B-cells and T-cells. Elsewhere they are associated with cells that line heart and blood vessels (*endothelial* cells), common connective tissue cells (*fibroblasts*), and some of the differentiating cells found in bone marrow (*stromal* cells).

To the above list might be added, as adjuncts, certain factors produced by immune cells, such as *lymphokines*, manufactured by lymphocytes, and *monokines,* made by monocytes and macrophages.[605] Lymphokines promote development and differentiation of natural killer cells (NKs), as well as B-cells and T-cells, etc., and participate in immune system modulation and systemic inflammation. Monokines regulate the function of other cells, such as lymphocytes. Among well known monokines are IL-1 and TNF (see above list and chart below).

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[599] Mak and Saunders, *The Immune Response*, 22, and see Table 2.2 (p. 23).
[600] For an introduction, see Hughes and Nibbs, "A Guide to Chemokines and Their Receptors."
[601] For an introduction, see Graber and Dhib-Jalbut, "Interferons." On their role in inflammation, see Kopitar-Jarala, "The Role of Interferons."
[602] For an introduction, see Akdis et al., "Interleukins (from IL-1 to IL-38)."
[603] See Sanjabi et al., "Anti- and Pro-Inflammatory Roles."
[604] For an introduction, see Atzeni and Sarzi-Puttini, "Tumor Necrosis Factor."
[605] For an introduction to both, see Klegerman and Plotnikoff, "Lymphokines and Monokines."

The following table offers some examples of cytokines.

CYTOKINE	Immune Cell Source	Targets (emphasizing immune system)	Function (emphasizing immune system)
IFN-α and IFN-β	Dendritic cells (DCs) Fibroblasts	all cells	defense against viral infection
IFN-γ	Natural Killer (NKs) T-cells (Th1) Cytotoxic T-cells (Tc)	Monocytes Lymphocytes	antiviral properties; immunoregulation; B-cell differentiation
IL-1α and IL-1β	Neutrophils Monocytes Macrophages Dendritic cells B-cells (some) Fibroblasts	Neutrophils B-cells T-cells (helper: Th) Fibroblasts Endothelial cells Epithelial cells	activate target cells; isolation of pro-inflammatory proteins
IL-2	Dendritic cells Mast cells Natural Killer cells T-cells (helper: Th1)	Natural Killer cells Monocytes B-cells T-cells (Th & Tc)	Activation and proliferation of target cells NKs & T-Cells
IL-3	Natural Killer cells T-cells (helper)	Macrophages Mast cells Natural Killer cells Stem cells	Activation of Basophils & Eosinophils
IL-4	Natural Killer cells Mast cells T-cells (Th2)	Eosinophils Macrophages Mast cells B-cells T-cells (NKT)	Division and differentiation of cells; role in inflammation
IL-5	T-cells	Eosinophils Mast cells B-cells	Differentiation of myeloid cells
IL-6	Macrophages B-cells (some) T-cells (Th2)	B-cells T-cells	Differentiation and activation of target cells (T-cell); antibody secretion
IL-8	Macrophages	Granulocytes (e.g., Neutrophils) T-cells	Chemotaxis (movement in response to chemical stimulus)
TGF-β	Eosinophils Macrophages Endothelial cells Fibroblasts	Eosinophils Macrophages Monocytes Neutrophils, etc.	regulatory and inflammatory activity (balancing)
TNF- α and TNF- β	Neutrophils Monocytes Macrophages T-cells	Macrophages Endothelial (ECs) Fibroblasts	Inflammation; produce other cytokines; cytotoxicity

Please note that the preceding chart, drawn from a number of sources,[606] is selective (thus it is partial and not comprehensive), and meant only to be broadly illustrative about cytokines. In listing sources and targets an emphasis has been placed on immune system cells, with innate system cells being listed before adaptive system cells.

Some of the cells listed above have a relationship to the immune system but without generally being listed as immune system cells. For example, the *endothelial* cells of the endothelium are sometimes termed 'conditional innate immune cells' and participate in both innate and adaptive immune system responses.[607] *Fibroblasts*, most noted for their presence in connective tissue but present in all tissues, have been called 'sentinel cells' that not only can recognize pathogens, but then recruit inflammatory cells via cytokines and growth factors.[608] *Stem* cells, of course, give rise to the specialized immune cells.

Interferons (IFNs) derive that name from their early gained notice of antiviral properties in 'interfering' (hence 'interfere' + particle suffice 'on') with viral replication. When secreted from infected cells they activate an innate immune response—including promotion of cytokine production and NK cell functions. The interferons have been divided into three classes (Types I, II, and III).[609] The IFN-α and IFN-β cells listed in the above chart are both Type I.

The chart above is dominated by *interleukins*. The name is derived from an early observation that such cells seem to communicate 'among' or 'between' (*inter-*) 'white cells' (*-leukins*). In the forty years after 1977, when IL-1 was identified, some 360,000 published scientific articles referred to them[610]—and interest since has hardly waned. These chemical signals have a variety of sources, targets, and functions, as the chart above shows. They are often discussed with respect to inflammation.

As is obvious, many immune cells have robust roles as sources and targets of cytokines. Let us use just one example. In the innate immune system *macrophage cytokines* are secreted in response to inflammatory stimuli. The cytokines released include chemokines, interleukins (IL-1, IL-6, IL-8, IL-12) and tumor necrosis factor (TNF). Such chemicals can induce increased vascular permeability—a greater outflow of fluid associated with the progress of inflammation. They also recruit immune cells to the site of infection or tissue damage. At the systemic level such actions are experienced as fever. When these cytokines are released in appropriate levels they are beneficial, but when the response is exaggerated the result can be harmful—even fatal.[611]

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[606] Information compiled from a number of sources, importantly including Mak and Saunders, *The Immune Response*, 23, Adkis, et al., "Interleukins," and Sino-Biological (https://www. sinobiological.com/resource/cytokines/cytokines-types).

[607] Mai et al., "An Evolving New Paradigm: Endothelial Cells—Conditional Innate Immune Cells."

[608] Bautista-Hernández et al., "Fibroblasts."

[609] Kopitar-Jerala, "The Role of Interferons," 2.

[610] Adkis, et al., "Interleukins," 984.

[611] Duque and Descoteaux, "Macrophage Cytokines."

Three Critical Immune System Defensive Activities

The innate immune system must quickly engage critical defensive actions:[612]

1. *Recognize the danger*—a task assigned to pattern recognition receptors (PRRs).
2. *Initiate a cascade of signaling along host pathways*—something accomplished by innate immune signaling pathways.
3. *Produce proteins to inhibit virus replication*—a job well-suited to type I interferons (IFNs).

Implicit in these actions is also a willingness to cut the body's losses to protect the whole organism. This is done by killing damaged host cells as needed.

1. Recognize the Invader

The first task employs germline encoded *pattern recognition receptors* (PRRs)—specialized proteins that act as sensors able to recognize specific molecular patterns (structures on a pathogen).[613] The phrase 'germline encoded' is important. It means that the composition of these receptors comes from genes expressed in fixed fashion generation after generation.[614] This is mixed news; on the one hand, it limits receptor specificity (what the receptor will interact with), but on the other hand, because PRRs recognize relatively fixed features ('conserved regions') of what they respond to, and these are widely shared by many kinds of pathogens, they remain adept at recognizing enemies of body health.

PRRs can be divided into five families (listed alphabetically):[615]

PRR Family	What They Recognize
Absent in melanoma-2 like Receptors (AIM-2s)	bacteria
C-type Lectin Receptors (CLRs)	glycans, fungi
Nucleotide-binding oligomerization domain (NOD)-like Receptors (NLRs)	bacterial, fungal, parasitic and viral PAMPs
Retinoic acid-inducible gene 1 (RIG-1)-like Receptors (RLRs)	viral RNA
Toll-like Receptors (TLRs)	microbial glycans, nucleic acids, proteins, etc.

[612] The following points are briefly described in Coleman, "Determining How Coronaviruses Overcome the Interferon and Innate Immune Response," 223–24 (though 'invader' is used instead of 'danger'); elaboration is found in Linares-Fernández et al., "Tailoring mRNA Vaccine."

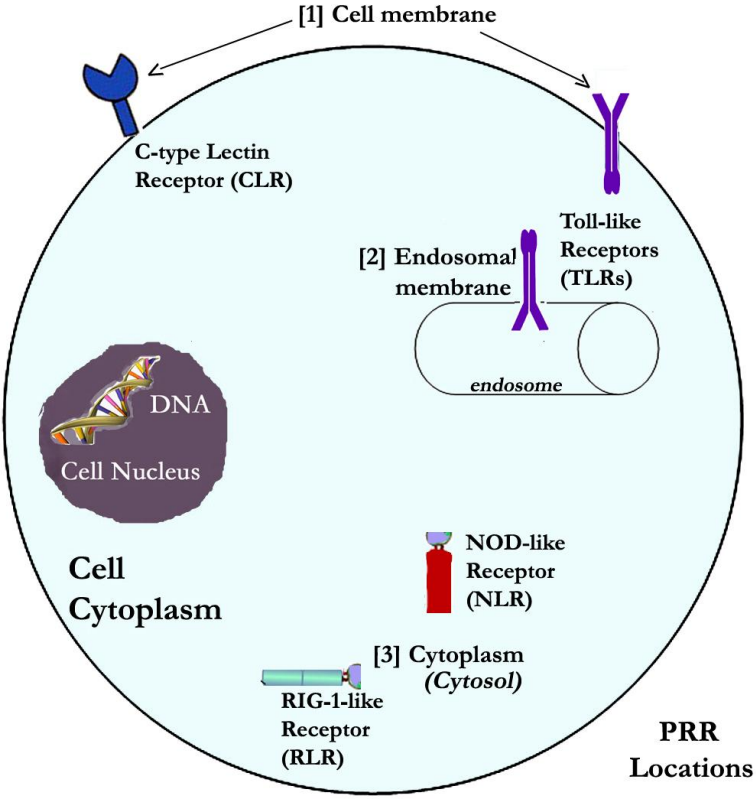
[613] For a general introduction on the matter, see Li and Wu, "Pattern Recognition Receptors in Health and Diseases," or Kumagai and Akira, "Identification and Functions of Patter-Recognition Receptors."

[614] Or as Mak and Saunders, *The Immune Response*, 85, put it, "the amino acid sequences of PRRs are derived from genes expressed inflexibly in the germ line." Akira, Uematsu, and Takeuchi, "Pathogen Recognition and Innate Immunity," write (p. 783), "PRRs are germline encoded, nonclonal, expressed on all cells of a given type, and independent of immunologic memory."

[615] Many sources count only four—those beneath AIM-2s, which are the most recently identified.

Although PRRs are placed as part of the innate immune system response (due to their evolutionary appearance prior to adaptive immunity),[616] some also induce signals that lead to activation of the adaptive immune system.[617] They thus have a role in the entire immune system response. They are found on various cells of the immune system (e.g., dendritic cells; macrophages) and elsewhere (e.g., epithelial cells).

PRRs might be better called *pattern recognition* ***molecules*** (PRMs) because they not only can be expressed by a variety of cell types, but also can be found *within* a cell as well as along its surface, where they act like a traditional receptor, recognizing and binding to particular parts of non-self objects.[618] PRMs are found along the cell membrane and inside the cytoplasm, as shown below.



Moreover, they are also found in secreted forms in the bloodstream and the fluid between cells and blood vessels (*interstitial* fluids).[619]

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[616] Amarante-Mendez et al., "Pattern Recognition Receptors," 1.
[617] Palm and Medzhitov, "Pattern Recognition Receptors and Control of Adaptive Immunity." Also, Amarante-Mendez et al., "Pattern Recognition Receptors," 2.
[618] Mak and Saunders, *The Immune Response*, 89.
[619] Amarante-Mendez et al., "Pattern Recognition Receptors," 1.

Absent in melanoma-2 like Receptors (AIM-2s)

Absent in melanoma-2 like Receptors (AIM-2s) are the most recently identified type of PRR. They recognize intracellular DNA, bind to it, and initiate a process promoting the formation of inflammasomes (discussed below).[620] They have been particularly linked to autoimmune diseases.[621]

C-type Lectin Receptors (CLRs)

C-type Lectin Receptors (CLRs) derive their name from calcium dependent (*C-type*) proteins that bind to carbohydrates (*lectins*). CLRs share at least one *carbohydrate recognition domain* (CRD), which determines the carbohydrate specificity of the CLR.[622] However, over time it became evident some CLRs lack coordinated calcium binding or carbohydrate specificity, and they are better termed *C-type lectin-like receptors*, each with at least one C-type lectin-like domain (CLRD). CLRs recognize both PAMPs and DAMPs.[623] Not all CLRs function as PRRs.

CLRs are found with monocytes, macrophages, and dendritic cells of the immune system, and outside with epithelial and endothelial cells. They principally utilize CRDs to recognize pathogen *ligands* (from Latin *ligere*, 'to bind'), to which they attach, internalize the pathogen, and then commonly degrade it via cell lysosomes (a process discussed elsewhere in this volume).[624] Their extensive range for what they recognize as well as their many and diverse roles have led them to be characterized as a 'super family' of proteins.[625] The following illustration offers an overview.

C-Type Lectin Receptors (CLRs)--a Type of Pattern Recognition Receptor (PRR)

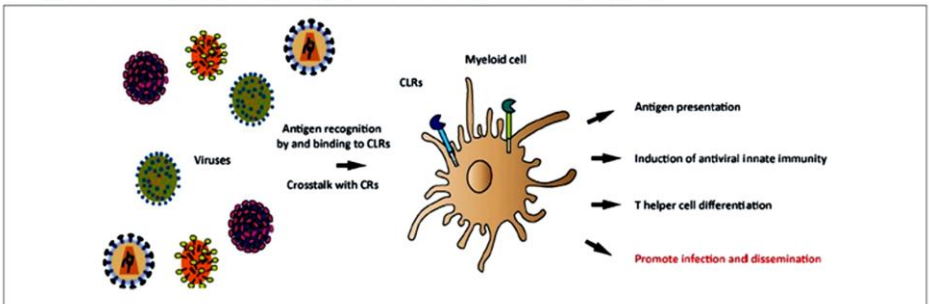

FIGURE 1 | Recognition of viral antigens by C-type lectin receptors (CLRs) and induction of antiviral immune responses. Various CLRs on antigen-presenting myeloid cells recognize a plethora of viruses through their carbohydrate-recognition domains (CRDs) and subsequently induce a tailored immune response, depending on the specific CLR and viral antigens. Viral antigens that trigger the CLR can modulate myeloid cell functions, thereby affecting antigen presentation, antiviral innate immune responses, and T helper differentiation. Once viral antigens are recognized by CLRs, crosstalk between CLRs and complement receptors (CRs) can occur, thereby further shaping the antiviral immune response. Additionally, CLRs play a role in viral recognition, internalization, and dissemination.

Illustration courtesy of Marta Bermejo-Jambrina, Julia Elder, LeanneC. Helgers, Nina Hertoghs, Bernadein M. Nijmeijer, Melissa Stunnenberg, and TeunisB. H. Geijtenbeek, "C-Type Lectin Receptorsin Antiviral Immunity and ViralEscape." *Frontiers in Immunology* 9 (Mar. 2018) art. 590. doi: 10.3389/fimmu.2018.00590. Made available by Creative Commons Attribution (CC-BY) License (https://creativecommons.org/licences/by/4.0/legalcode). The illustration appears on p. 2. No alternations have been made.

[620] Li and Wu, "Pattern Recognition Receptors," 7.
[621] See, for example, Caneparo et al., "The Absent in Melanoma 2-Like Receptor."
[622] Geijtenbeek and Gringhuis, "Signalling through C-type Lecting Receptors," 465.
[623] Ganguly, Kishore, and Madan, "Interplay between C-type Lectin Receptors," 4210–11.
[624] Bermejo-Jambrina et al., "C-type Lectin Receptors."
[625] Brown, Willment, and Whitehead, "C-type Lectins in Immunity and Homeostasis."

Nucleotide-binding oligomerization domain (NOD)-like Receptors (NLRs) are intracellular PRRs that function as sensors in cell cytoplasm. The NLRs are principally expressed by immune cells (*antigen-presenting cells* (APCs) such as macrophages and dendritic cells), but also can be found in epithelial and mesothelial cells. In humans, NLRs constitute a family of 22 proteins. These protein family members have been grouped into one or another of four subfamilies (NLRA, NLRB, NLRC, NLRP), and a one family member outlier (NLRX). Each of these groups is characterized by a particular kind of domain organization.[626]

Structurally, they look like this:

N-Terminal Domain	**Central NOD Domain**	**C-Terminal Domain**
↓	↓	*leucine-rich repeat (LRR)*
protein-protein interaction	*mediates self-oligomerization occurring during activation*	↙ ↘
<u>defined by one of following:</u> caspase recruitment domain (CRD); pyrin domain (PYD); acidic transactivating domain; or, baculovirus inhibitor repeat (BIR)[627]		*agonist sensing* | *ligand binding*

Interestingly, it has been observed that the architecture of NLRs resembles that found in a subset of plant disease-resistant genes involved in hypersensitive responses against virulent plant pathogens.[628]

NLRs in mammals are divided into four subfamilies based on variations found in the N-terminal domain. The C-terminal domain is responsible for ligand binding with a pathogen, which results in changes that expose the N-terminal domain so that it can interact with adaptor proteins or effectors and form an *oligomeric complex*—a larger structure leading eventually to inducing inflammation to control infection or endogenous damage.[629]

NOD1 and NOD2—probably the most remarked upon NLRs—sense bacterial molecules produced during the synthesis and/or degradation of peptidoglycan.[630] These NLRs have been associated with a range of autoimmune disorders (e.g., inflammatory bowel disease (IBD)). The NLRP subfamily is noted for its role in forming inflammasomes (discussed later in the chapter).[631]

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[626] Chen et al., "NOD-like Receptors," 366.
[627] Table utilizes Chen et al., "NOD-like Receptors," 366, and Zhong, Kinio, and Saleh, "Functions of NOD-like Receptors in Human Diseases," 1. Also see Inohara and Núñez, "The NOD."
[628] Kanneganti, Lamkanfi, and Núñez, "Intracellular NOD-like Receptors," 549, following Inohara and Núñez, "The NOD."
[629] Zhong, Kinio, and Saleh, "Functions of NOD-like Receptors in Human Diseases," 1.
[630] Kanneganti, Lamkanfi, and Núñez, "Intracellular NOD-like Receptors," 551.
[631] Heim, Stafford, and Nachbur, "NOD Signaling and Cell Death," 2.

Retinoic acid-inducible gene 1 (RIG-1)-like Receptors (RLRs) are found in the cytoplasm of most cell types, meaning they are widespread. Retinoic acid, made from vitamin A, promotes cell growth and development. RIG-1 is encoded in humans by gene DDX58. Family members include *retinoic acid-inducible gene 1* (RIG-1), *melanoma differentiation-associated gene 5* (MDA5), and *laboratory of genomics and physiology 2* (LGP-2). Of these, the first two seem to receive more attention and, because they recognize coronaviruses, are of more interest to us here.

RLRs are a fundamental first line of defense against viruses and have been found to recognize almost all major virus families (including the coronaviruses). They are key players in antiviral response, particularly through increased expression (i.e., 'transcriptional induction') of type I interferons (IFN).[632]

The basic structure for all RLRs has two domains (a central helicase domain and a carboxyl-terminal domain (CTD)), but our two family members of interest—RIG-1 and MDA5—add two amino-terminal caspase activation and recruitment domains (CARDs).[633] It can be portrayed this way:

2 Caspase Activation and Recruitment Domains (CARDs)	**Central Helicase Domain**	**C-Terminal Domain** (CTD)
facilitate signal transmission ('mediate downstream signal transduction)	*with CTD, works to*	*detect viral RNA*

But we can be even more specific with respect to RIG-1 and MDA5.

Structure of 2 RLRs

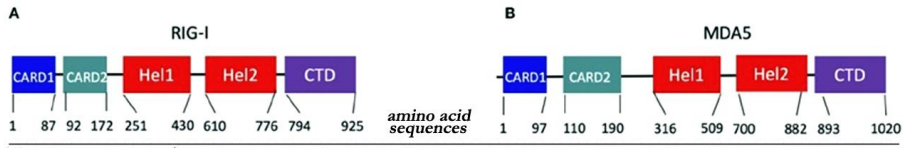

Illustration courtesy of Morgan Brisse and Hinh Ly, "Comparative Structure and Function of the RIG-I-Like Receptors: RIG-I and MDA5." *Frontiers in Immunology* 10 (July, 2019) art. 1586. doi: 10.3389/fimmu.2019.01586. The image is excerpted from Fig. 3 (p. 5). Material is available by Creative Commons Attribution (CC-BY) License (https://creativecommons.org/licenses/by/4.0/). The original image has been altered by excerpting just portions A and B, adding a title above, and supplying the words "amino acid sequences."

RLRs can recognize both exogenous dangers like RNA viruses (PAMPs), and also endogenous dangers, like cellular RNAs that are unusual, mislocalized, or misprocessed.[634]

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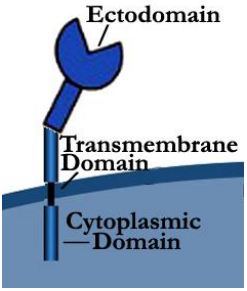
[632] Rehwinkel and Gack, "RIG-1-like Receptors."
[633] Rehwinkel and Gack, "RIG-1-like Receptors," 537.
[634] Rehwinkel and Gack, "RIG-1-like Receptors," 538; Roh and Sohn, "Damage-Associated Molecular Patterns in Inflammatory Diseases," 3 (Table 2).

Toll-like Receptors (TLRs) were identified at the end of the 20th century—a discovery significant enough to be honored with the Nobel Prize in Physiology or Medicine in 2011.[635] The label 'Toll' was inspired by the observation that these proteins resemble that coded by the 'toll' gene—from a German expression for something fantastic (*Toll!*), the response by a researcher in the early 1980s while dissecting genetic pathways in the common fruit fly (*Drosophilia melanogaster*).[636] They are distinguished from other PRRs as being the most ancient and possessing the most extensive spectrum of pathogen recognition.[637]



TLRs are found in innate immune system cells (dendritic cells; macrophages), but also nonimmune cells (epithelial cells; fibroblasts). TLR 'families' are divided into 'subfamilies' that are distinguished from each other by whether the receptor is along the cell surface or within the cytoplasm (specifically, the endosome). Whether spanning the cell membrane or that of an endosome, TLRs display three domains (see illustration at left).

The domain outside the cell (*extracellular*)—the *ectodomain*—is characterized by a *leucine-rich repeat* (LRR) *domain*. LRRs are important in recognizing the portion of a pathogen the TLR binds to (the *ligand*) and also in signal transmission through the cell ('transduction').[638] Thus the ectodomain is the portion that recognizes dangerous foreign materials called PAMPs (discussed in the next section), then participates in the signaling operation of the TLR. The *transmembrane domain* serves to anchor the receptor. The *cytoplasmic domain* (formally known as *cytoplasmic Toll/IL-1 receptor (TIR) domain*)—the portion found inside the cell—is responsible for initiating signaling,[639] a process we shall examine further in discussing signaling pathways.

Ten mammalian TLRs are known—and each appears to have a distinct function. The binding molecules (*ligands*) these TLRs recognize number in the dozens. Though these ligands are diverse in origin and structure, they seem to show four themes: [1] they are PAMPs that signal infection; [2] most (perhaps all) TLRs can recognize diverse ligands even though they are structurally unrelated; [3] to recognize ligands some TLRs require accessory proteins; and, [4] TLRs appear to bind directly to their ligands—and are thereby PRRs.[640]

TLRs are important in many activities, including inducing inflammation and interacting with the adaptive immune system.

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[635] O'Neill, Golenbock, and Bowie, "The History of Toll-like Receptors."
[636] Chtarbanova and Imler. "Microbial Sensing by Toll Receptors," 1734.
[637] Nie et al., "Toll-like Receptors," 1.
[638] Medhitov, "Toll-like Receptors and Innate Immunity," 136.
[639] Kawasaki and Kawai, "Toll-like Receptor Signalling Pathways," 1.
[640] Medhitov, "Toll-like Receptors and Innate Immunity," 139.

The table earlier in this section broadly characterizes what various PRRs recognize—pathogenic structures in bacteria, viruses and the like. But it is more precise to say that PRRs recognize *two* groups of molecular patterns:

- *pathogen-associated molecular patterns* (PAMPs); and.
- *damage-associated molecular patterns* (DAMPs).

The difference is simple enough. PAMPs, as the first word states, are particular features of the molecular make-up of an invading pathogen (e.g., a virus). They are *exogenous*—i.e., originating from outside the body. DAMPs, on the other hand, are *endogenous*—molecules inside the body released by damaged host cells. So the one (PAMPs) refers to molecular patterns belonging to foreign bodies and the other (DAMPs) to molecular patterns produced by the human body.

Pathogen-associated molecular patterns (PAMPs) are distinguished by three important features. First, these patterns are invariant among entire classes of pathogens (meaning they have wide applicability to the immune defense system). Second, they are structures essential to the pathogen's survival; target them in immune defense and the pathogen can be overcome. Third, they are distinguishable as 'non-self,' meaning the immune system readily recognizes them as signaling danger.[641]

PAMPs include carbohydrates (*glycans*), proteins, lipids, lipoproteins, and nucleic acids.[642] However, of special interest to us are PAMPs associated with viruses. These include surface glycoproteins (important to viral attachment to host cells), and the DNA or RNA of various viruses. Unfortunately, as Trine Mogensen, a specialist in infectious diseases, points out, "The outcome of virus-mediated PRR activation can range from an antiviral response that efficiently clears the infection to the establishment of a cellular environment that favors viral replication and spread."[643]

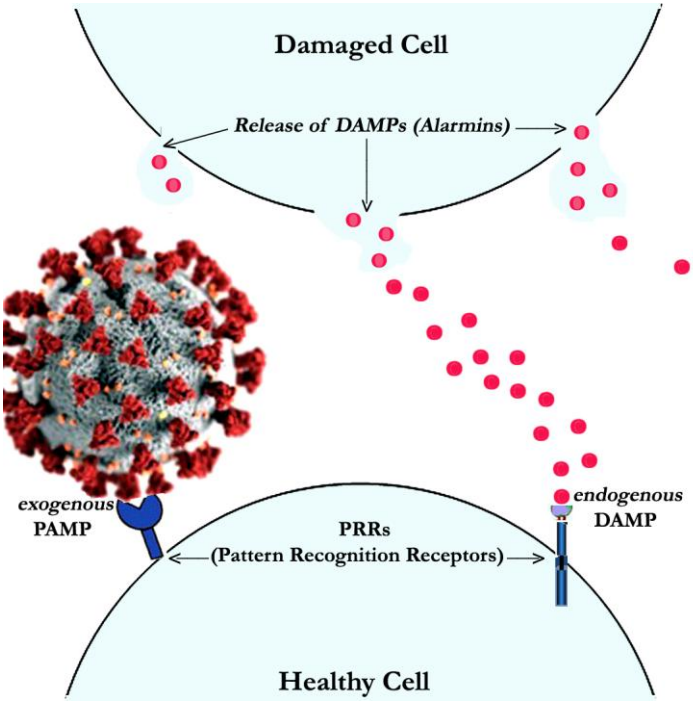
The idea of *damage-associated molecular patterns* (DAMPs) was born out of the observations and arguments of immunologist Polly Matzinger, who in the mid-1990s proposed thinking of the immune system as more about recognizing *danger* than about recognizing a difference between self/non-self. She noted, "Any internal molecule that is normally not secreted, be it cytoplasmic, nuclear, or membrane, could act as an activator of dendritic cells when released. Thus any cell damage would be noticed. . . ."[644] Such observations gave rise to the recognition of DAMPs,[645] or as some prefer, *alarmins*—"endogenous molecules that

[641] These characteristics are listed by Mogensen, "Pathogen Recognition," 241.
[642] Ramírez Hernández et al., "The Role of the SARS-CoV-2 S Protein," 166. 'Toll-like receptor' is a name that has nothing to do with a 'toll-like' function, but refers to their similarity to the Toll gene in fruit flies (*drosophilia*).
[643] Mogensen, "Pathogen Recognition," 245.
[644] Matzinger, "Tolerance, Danger, and the Extended Family," 1037.
[645] Amarante-Mendez et al., "Pattern Recognition Receptors," 2.

signal tissue and cell damage."[646] Sometimes DAMPs is used as an umbrella term covering PAMPs and alarmins, and sometimes—as in this volume--as a label for a general class recognized by PRRs that is distinct from PAMPs.

Whether one prefers to use the term alarmins or speak instead of DAMPs, certain characteristic features have been noted for them. First, when a cell dies unexpectedly (i.e., not from *apoptosis*—normal, programmed cell death), DAMPs are rapidly released. Second, immune system cells can be induced to produce and release them without dying. Third, DAMPs 'recruit'—i.e., enlist for a specific task—innate immune cells that are receptor-expressing, and then activate them. Fourth, they help restore homeostasis by promoting reconstruction of what was lost due either to direct cell injury or secondarily because of inflammation.[647]

A number of putative DAMPs (or alarmins) have been identified, including *annexin* (ANX), which are calcium-dependent cellular proteins; *cathelicidin* (CAMP), which are short peptides and a DAMP sometimes produced by immune system cells; and, defensins, another small peptide and most famous as antimicrobial in nature.[648] The illustration below shows PRRs, PAMPs, and DAMPs.



[646] Bianchi, "DAMPs, PAMPs and Alarmins: All We Need to Know about Danger," 1. For how the term 'alarmin' developed, see Yang, Han, and Oppenheim, "Alarmins and Immunity."
[647] Bianchi, "DAMPs, PAMPs and Alarmins: All We Need to Know about Danger," 2.
[648] For a more complete list see Bianchi, "DAMPs, PAMPs and Alarmins: All We Need to Know about Danger," 2 (Table 1); on defensins, see Yang, Han, and Oppenheim, "Alarmins and Immunity."

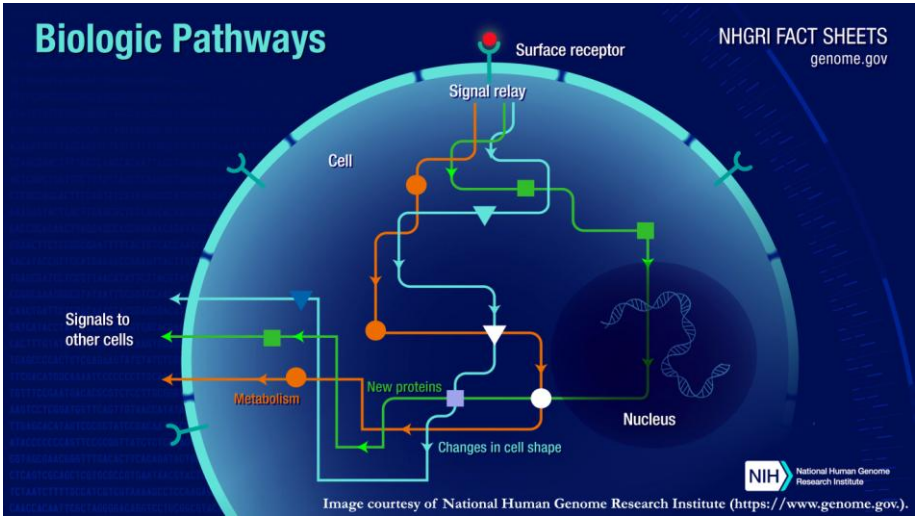
2. Initiate Signaling of the Immune System

The pattern recognition receptors (PRRs), having accomplished the first task of recognizing the danger posed either by exogenous pathogens or endogenous damaged cells, then use *signaling pathways* to indicate that defensive action is needed. Various innate system signaling pathways have drawn considerable attention. We shall briefly consider a few of them.

Pathways	Brief Description
C-type Lectin receptor (CLR) *2 broadly differentiated kinds (mannose receptor (MR) or fucose-specific); 17 subgroups representing more than 1,000 proteins in vertebrates*	Recognition of PAMPs or DAMPs prompts signaling cascades that elicit production of inflammatory mediators. Activation may induce signaling either directly (through ITAm-like motifs) or indirectly (associating with ITAm containing adaptor molecules). Some CLRs collaborate with TLRs in signaling.
NOD-like receptor (NLR) [NOD1 and NOD2] *22 members in 4 subfamilies: NLRA, NLRB, NLRC, NLRP—plus outlier NLRX*	Upon recognition and binding to PAMPs they undergo chemical conversion and interact with adaptor protein RIP2 (Receptor-interacting-serine/threonine-protein kinase 2) to activate pathways. These PRRs also are involved in autophagic response to invading bacteria.
RIG-1-like receptors (RLRs) *family includes RIG-1. MDA5, and LGP-2*	Activated RLRs interact with MAVS resulting in formation of a MAVS Complex, which engages kinases and leads to a series of changes resulting in activation of Type I interferon genes.
Toll-like receptor (TLR) 2 subfamilies: *cell surface* (TLRs 1, 2, 4, 5, 6, 10) *intracellular* (TLRs 3, 7, 8, 9)	Upon binding to PAMPs undergo changes in conformation that lead to recruiting TLR domain-containing adaptor proteins, which recruit other elements that produce various effects, including leading to synthesis of proinflammatory cytokines.
Inflammasome NLRs (NLRP1, NLRP3, NLRP6, NLRP7, NLRC4) AIM-2	As noted at left, these utilize various PRRs, most notably NLRs. These receptors function as sensors or adaptors to form 'inflammasome'—a complex of large proteins responsive to a variety of stimuli (PAMPs and DAMPs); the complex then initiates immune responses inflammatory in nature.
Type I Interferon	Induced both by DNA and RNA viruses, various RLRs recognize viral components and activate adaptor proteins, which are engaged to enlist other factors leading to a signaling that elicits immune response.[649]

[649] The table idea started with Cui et al., "Mechanisms and Pathways of Innate Immune Activation," 3271–73. This was built upon by use of other sources. For another presentation, see Roh and Sohn, "Damage-Associated Molecular Patterns in Inflammatory Diseases," 3 (Table 2).

The basic idea of a signaling pathway is simple enough. Having recognized some form of danger, whether from a foreign pathogen (exogenous) or from a problem within the body (endogenous), pattern recognition receptors (PRRs) now need to alert the immune system. Just as a person in trouble might dial 911, thus activating a signal along telephone lines to an operator who can initiate a proper response by the right actors, so a PRR engages a signaling process along one or another 'pathway.' The National Human Genome Research Institute (NHGRI) offers a solid definition: "A biological pathway is a series of actions among molecules in a cell that leads to a certain product or a change in the cell."[650] Their accompanying illustration may help provide a better overview.



A bit further on we shall illustrate this basic idea more specifically with respect to PRRs. But for now this general concept needs some elaboration by examining various PRR families and some of the pathways they employ.

While generalizations tend to mislead, especially when taken to apply to all specific instances, we can say that PRR pathways *tend* to share certain features. First, the PRR senses danger. Then they precipitate molecule chains using that workhorse of the cell—proteins. Adaptor proteins, in particular, are important. Just as in electronic applications, 'adaptors' connect together different pieces in a signaling pathway. By hooking things together, platforms—biological complexes of molecules—can boost signaling strength as the message is carried down the line to where it needs to reach. At some point the signal reaches molecules that trigger specific actions, such as initiating inflammation. Broadly described, the point is to sound the alarm in an effective fashion so that the immune system mounts a speedy and effective response.

[650] NHGRI, "Biological Pathways Fact Sheet" (https://www.genome.gov/about-genomics/fact-sheets/Biological-Pathways-Fact-Sheet), accessed Feb. 16, 2022.

The first listed set of pathways in the table at the start of this section is associated with the PRR type called *C-type Lectin receptors* (CLRs). As noted earlier, this is a very large family. Not surprisingly, then, there are various pathways different CLRs may utilize for signaling.[651] CLRs that function as PRRs and use one or another pathway to signal the need for immune system response mostly belong to two subfamilies.

The first is part of CLR family group V and known as *Dectin-1*. Key to its signaling action is the *immunoreceptor tyrosine-based inhibition motif* (ITIM) or the *hemi-immunoreceptor tyrosine-based activation motif* (hemi-ITAM, or hemITAM) in its cytoplasmic tail. The key ideas of 'inhibition' and 'activation' are underscored to highlight their effect, but more basic is 'immunoreceptor tyrosine-based motif.' That central idea—applying to both ITIM and ITAM—refers to employing the amino acid tyrosine to a function specific to immune receptors, namely, activating them (turning them 'on') or inhibiting them ('off'). ITAM-bearing receptors typically result in cell activation while ITIM-bearing receptors usually inhibit cells. Both ITIMs and ITAMs are changed to facilitate signaling (*phosphorylated*) by certain enzymes (*src family protein tyrosine kinases*); this renders them able to recruit molecules in the cell cytoplasm that have *Src Homology 2* (SH2) domains, thus linking receptor activation to cell signaling.[652]

Dectin-1 receptors and pathways include some that use ITIM—*myeloid inhibitory C-type lectin-like* (MICL) and *C-type lectin-like receptor 12b* (CLEC12B)—both of which recruit two phosphates (SHP-1 and SHP-2) important to signaling in order to negatively regulate ('inhibit') inflammatory responses. Others use hemi-ITAM—*C-type lectin-like receptor 2* (CLEC-2, also known as CLEC-1b), *C-type lectin-like receptor 9a* (CLEC9a), and *DC-associated C-type lectin 1* (Dectin-1)—which mediate signaling through *spleen tyrosine kinase* (Syk)-dependent pathways. Dectin-1 (also known as CLEC7a) uses hemi-ITAM in various pathways to induce or regulate many cellular responses, including cell degradation (*autophagy*) and ingestion of pathogens (*phagocytosis*). *Lectin-like oxidized low density lipoproteins (LDL) receptor-1*(LOX-1) induces signaling that also elicits a number of cellular responses, including production of cytokines and the activation of NALP3 inflammasome. Finally, the last member, *Melanin sensing C-type lectin receptor* (MelLec) has a mechanism of signaling that remains unclear.[653]

The other main subfamily is *Dectin-2* of group II in the CLR family of receptors, which includes PRRs Dectin-2, *macrophage C-type lectin* (MCL), and *macrophage-inducible C-type lectin* (Mincle). Each member has one or more *carbohydrate recognition domains* (CRDs) that make it possible to recognize danger and initiate

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[651] See, for example, Figure 1 in Hoving, Wilson, and Brown, "Signalling C-Type Lectin Receptors," 186, and Figue 2 in Bermejo-Jambrina et al., "C-type Lectin Receptors," 3.

[652] Daëron and Vivier, "Biology of Immunoreceptor Tyrosine-based Inhibition Motif-Bearing Molecules." On SH2 particularly, see Waksman, Kumaran, and Lubman, "SH2 Domains."

[653] Tone et al., "C-type Lectin Receptors of the Dectin-1 Cluster."

immune signaling. They do this either by using their own cytoplasmic domains, or by employing the immunoreceptor tyrosine-based activation motifs present in the *Fc receptor common gamma chain* (FcRγ).[654] The Fc receptor recognizes the *fragment crystallizable* (Fc) *region* of antibodies and is thus an antibody receptor that activates immune response.

NOD-like Receptor (NLR) Pathways

The pathways associated with the PRR type known as *NOD-like receptors* (NLRs)—specifically NOD1 and NOD2—can be activated either by PAMPs or by DAMPs. Once activated, NLRs activate inflammatory pathways—notably those associated with a protein transcription factor named *nuclear factor–kappa* B(NF-κB) and MAPKs.[655] Some NLRs *oligomerize*—they undergo a process in which polypeptide chains interact to form a large structure with a high degree of organization and stability. In this manner, some NLRs form 'inflammasome complexes' (see below). These complexes serve as platforms facilitating recruitment, cleavage, and activation of inflammatory *caspases*—proteins that act as enzymes, causing chemical reactions instrumental to causing inflammation.[656]

RIG-1-like Receptor (RLR) Pathways

The Rig-1-like receptors (RLR) play a starring role in antiviral defense by recognizing dangerous RNA. As they bind to a PAMP, either of the RLR family members RIG-1 or MDA5, interact with *mitochondrial antiviral signaling proteins* (MAVS). As the name indicates, MAVS are proteins localized to cellular mitochondria, those vital structures responsible for cell respiration (combining oxygen with nutrient materials) and producing energy. They are abundant in cell cytoplasm and thus, in addition to their role in cell metabolism, they provide an important platform for immune system signaling. While mitochondria are also involved with other PRRs (TLRs and NLRs) they are especially noted with RLRs, serving as key adaptor proteins.[657]

Interaction with MAVS results in a multilayered protein complex populated by diverse proteins. This complex then catalyzes the interaction between two serine/threonine kinases—one that plays an essential role in regulating inflammatory responses to viral infection (*inhibitor of nuclear factor kappa-B kinase subunit epsilon* (IKK+)) and *serine/threonine-protein kinase 1* (TBK1). This facilitates signal transmission (via phosphorylation) using *transcription factors interferon regulatory factors 3* and *7* (IRF3 and IRF7), which in phosporylated form (*p-IRF7* and *p -IRF3*) combine (*dimerize*), move to the nucleus, amd activate Type I interferon genes.[658] (See illustration, next page.)

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[654] Kottom et al., "Dectin-2 Is a C-type Lectin Receptor," 237, and Haider et al., "The Pattern Recognition Receptors Dectin-2, Minacle, and FcRγ," 2.
[655] Chen et al., "NOD-like Receptors," 366.
[656] On the relation of NLRs to inflammasomes, see Zhong, Kinio, and Saleh, "Functions of NOD-like Receptors in Human Diseases," 2 (esp. Figure 2).
[657] Refolo et al., "Mitochondrial Interactome."
[658] Brisse and Ly, "Comparative Structure and Function: Analysis of RIG-1-like Receptors," 2.

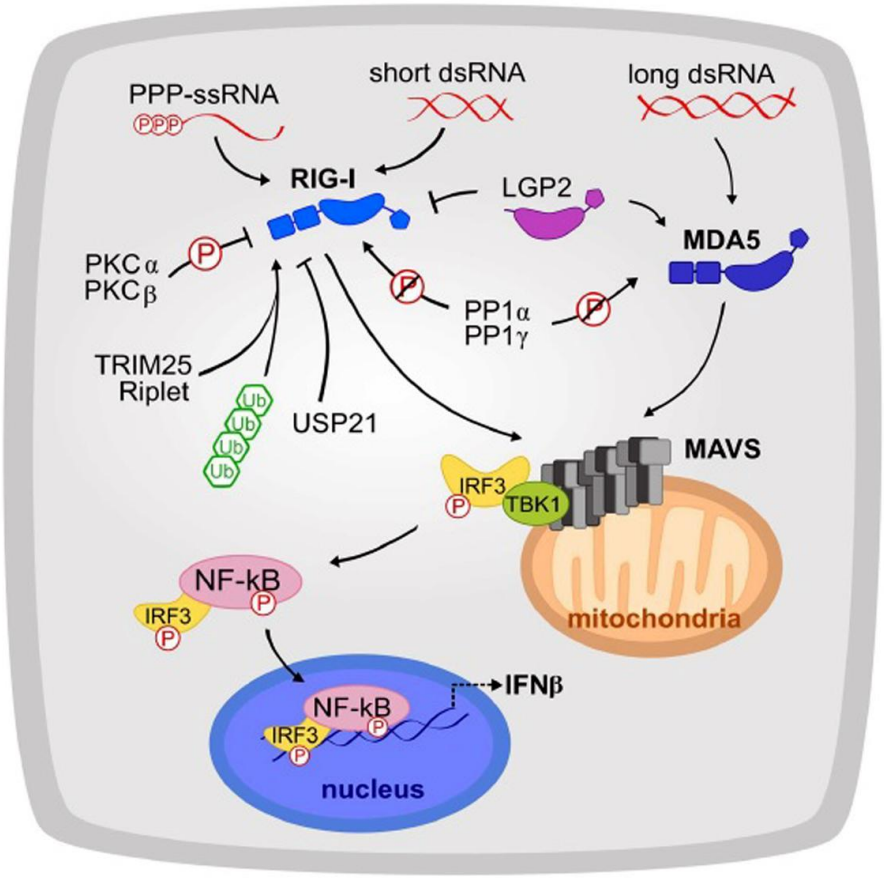
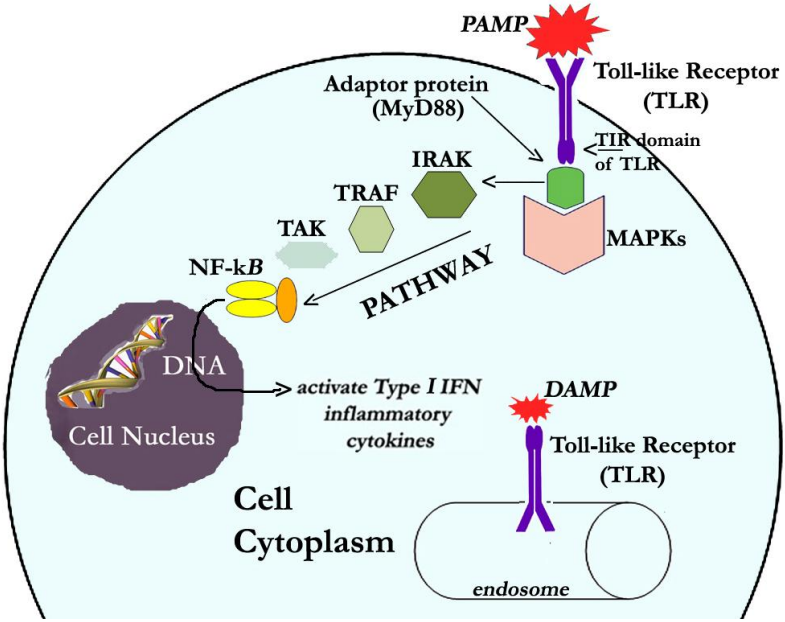


FIGURE 1|The RLR signaling pathway is shown. RIG-IandMDA5 recognize a complementary set of cytosolic viral dsRNA ligands.Their activation is tightly regulated by phosphorylation, ubiquitination, and host proteins such as LGP2. RIG-I and MDA5 signal to MAVS, which initiates the production of interferon signaling. Circled "P" indicates phosphorylation and slashed circled "P" indicates dephosphorylation.

Illustration courtesy of Stephanie Reikine, Jennifer B. Nguyen, and Yorgo Modis. "Pattern Recognition and Signaling Mechanisms of RIG-1 and MDA5." *Frontiers in Immunology* 5 (July, 2014) art 342. doi: 10.3389/fimmu.2014.00342. The material, found on page 2, has been made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/legalcode).

Toll-like receptors (TLRs) recognize PAMPs. The molecular patterns various TLRs recognize include microbial lipids, lipoproteins, proteins, and nucleic acids. The different TLRs recruit one or another of a set of TIR domain-containing adaptor proteins.[659] *Adaptor proteins*, as microbiologist and immunologist Daniel Flynn explains, "contain a variety of protein-binding modules that link protein-binding partners together and facilitate the creation of larger signaling complexes."[660] The task of the TIR domain-containing adaptor proteins is to initiate signaling pathways. The most notable of these adaptor proteins is *MyD88*—the MyD88 gene-encoded protein that connects proteins receiving signals from outside the cell to those inside the cell that relay signals. All TLRs use this adaptor and by it activate a protein complex (*NF-kappaβ* (NF-kβ)) and *Mitogen-activated protein kinases* (MAPKs) to induce inflammatory cytokine genes.[661] Additional players in a TLR pathway to inflammation might include *interleukin-1 receptor-associated kinases* (IRAKs), *TNF receptor associated factors* (TRAFs), and *transforming growth factor-β activated kinases* (TAKs), among others. The role of TLRs and their pathways in relation to inflammation most often is discussed with reference to microbial pathogens (PAMPs), but TLRs also can recognize endogenous dangers (DAMPs) and have been implicated in initiating and sustaining autoimmune diseases.[662] Here is a simplified illustration of this:



[659] Kawasaki and Kawai, "Toll-like Receptor Signalling Pathways," 1–2.
[660] Flynn, "Adaptor Proteins," 6270.
[661] Kawasaki and Kawai, "Toll-like Receptor Signalling Pathways," 2.
[662] Drexler and Foxwell, "The Role of Toll-like Receptors in Chronic Inflammation."

The general illustration above can be supplemented by the following one:

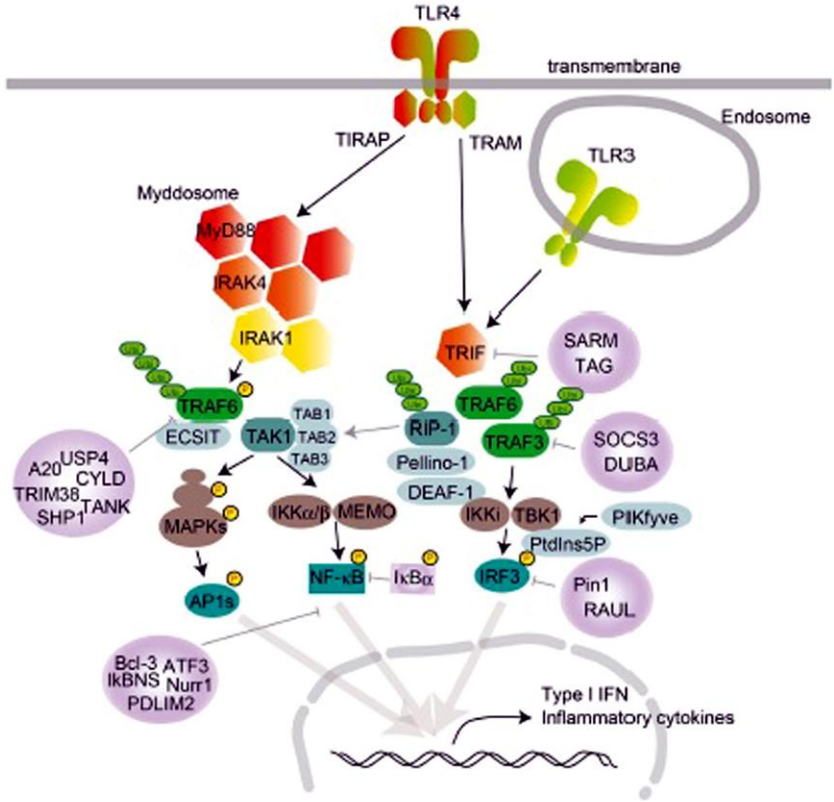


FIGURE 1|TLR signaling in cDCs, macrophages, and MEFs. TLR4 localize to the cell surface, and TLR3 localize in the endosome compartment. Homo- or heterodimer formation initiates signaling to the two major downstream adaptor proteins, MyD88 and TRIF. TIRAP conducts the signal from TLR4 to MyD88, and TRAM mediates the signal from TLR4 to TRIF. TLR engagement induces formation of the Myddosome, which is based on MyD88 and also contains IRAK1 and IRAK4. IRAK1 activation induces TRAF6 activation following K63-linked polyubiquitination on TRAF6 itself and TAK1. TAK1 activation leads to the activation of IKK complex-NF-κB and MAPKs. MAPK activation leads to AP1s transcription factor activation. TRAF6 promotes ECSIT ubiquitination, resulting in increased mitochondrial and cellular ROS generation. TLR engagement also induces TRIF activation following TRAF6 and TRAF3 recruitment. TRAF6 recruits RIP-1, which activates the TAK1 complex following MAPK activation. RIP-1 activation regulates ubiquitination by Pellino-1. Pellino-1 regulates IRF3 activation by binding to DEAF-1. TRAF3 recruits TBK1 and IKKi for IRF3 phosphorylation. PtdIns5P from PIKfyve facilitates complex formation between TBK1 and IRF3. Several negative regulators modulate TLR signaling, by inhibiting either signaling complex formation or ubiquitination. MyD88 is suppressed by S2825, NRDP-1, SOCS1, andCbl-b; TRIF is suppressed by SARM and TAG; TRAF3 is suppressed by SOCS3 and DUBA; and TRAF6 is suppressed by A20, USP4, CYLD, TANK, TRIM38, and SHP. NF-κB is suppressed by Bcl-3, IkBNS, Nurr1, ATF3, and PDLIM2, while IRF3 activation is negatively regulated by Pin1 and RAUL.

(Preceding illustration courtesy of Takumi Kawasaki and Taro Kawai, "Toll-like Receptor Signaling Pathways." *Frontiers in Immunology* 5 (Sept., 2014) art 461. doi: 10.3389/fimmu.2014.00461. The material, found on page 3, has been made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/legalcode). (*Also see the complementary illustration on page 4.*))

In examining this more complex illustration it may be easier to see why we have kept to the shallow end of the pool. The expert knowledge reflected above is not needed for the rest of us to achieve a basic grasp of an important process. But looking a little deeper like this should help us appreciate more how complex a process this business of the body is. The 'pathway,' remember, is comprised of a series of actions among molecules in a cell that leads to a certain product or a change in the cell. For those interested in exploring this matter further, there are many articles and illustrations available on the World Wide Web that collectively reflect the variety of TLR pathways. And TLR pathways are just a part of the larger picture we are painting of PRRs doing their job of recognizing danger and then engaging an alert system to mobilize the body's defenses.

But before we move on, let us linger a moment on this more complex illustration in order to focus on two matters to which we will next turn. Both are at the bottom of the illustration. The grey bordered area is the cell nucleus. The TLR pathway has led to activation of changes inside the nucleus.

First, note the term 'inflammatory.' The importance of inflammation was highlighted in a section in the previous chapter. Inflammation is a natural result following from cell injury or damage caused by PAMPs or DAMPs. The inflammation, when all goes well, destroys pathogens and promotes healing. In essence, inflammation [1] removes injurious stimuli and [2] initiates the healing process.[663] An important part of the whole inflammatory response is the inflammasome, to which we will turn next.

Second, the word 'inflammatory' is joined to 'Type I IFN inflammatory cytokines.' That topic will be the last one in this section. IFNs—interferons—are a kind of cytokine (a group we examined earlier in this chapter). The TLRs we are briefly considering here are often involved in the process that produces interferons, which are important antiviral agents. They are also part of the inflammatory response.

The reason these observations are merited here is simple: in looking at myriad trees it is easy to lose sight of the forest. For example, TLRs are not divorced from either inflammasomes or interferons. There is what has been characterized as 'cooperation' between TLRs and NLR inflammasomes.[664] With respect to interferons (IFNs), TLRs commonly initiate the process that results in interferons being produced. All the many players and processes we are examining are part of a larger and very sophisticated whole we must strive to see as such.

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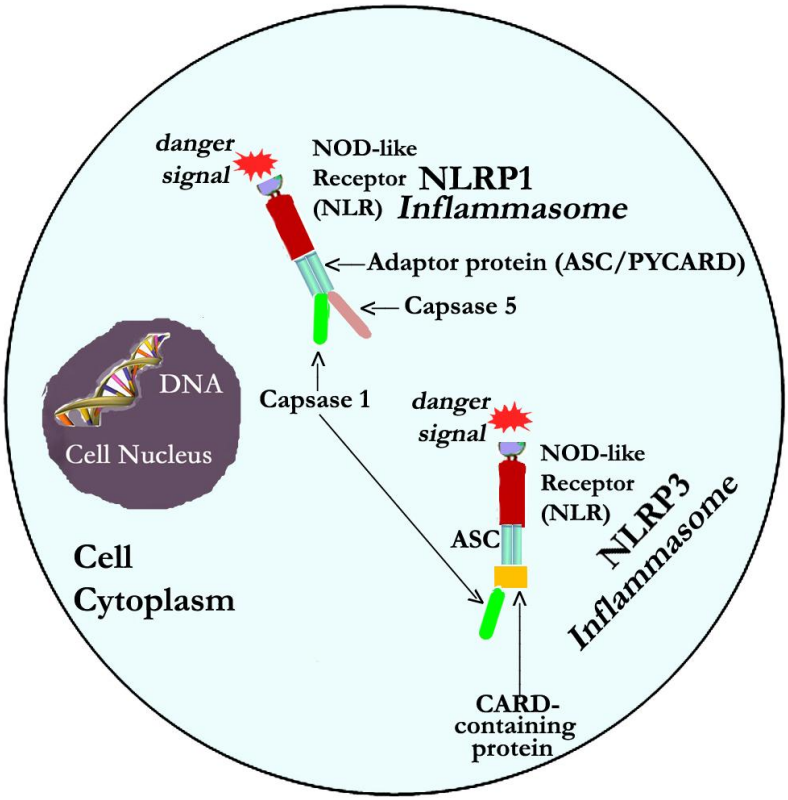
[663] Chen et al., "Inflammatory Responses," 7204.

[664] See, for example, Hanamsagar, Hanke, and Kielian, "Toll-like Receptor (TLR) and Inflammasome Actions."

Near the beginning of the 21st century, biochemist Jurg Tschopp employed the term *inflammasome* (literally, an 'inflammatory' (*inflanna-*) 'body' (*-some*)) to label a biological complex that senses danger and initiates an inflammatory response. This system was quickly tied to the kind of PRRs known as NOD-like receptors (NLR; see chart, p. 206). An *inflammasome pathway* (the NLRP1) was recognized with these constituent parts: [1] a NLR protein (NLRP1); [2] an adaptor protein (*apoptosis-associated speck-like protein containing CARD* (ASC, also known as PYCARD)—an element instrumental in creating larger signaling complexes; and [3] two caspase proteins (*caspase 1* and *caspase 5*) important in triggering inflammation that leads to cell death (*pyroptosis*). Almost immediately thereafter a NLRP3 inflammasome was identified (with NLRP3, ASC, a *caspase activation and recruitment domain* (CARD)-containing protein, and caspase 1). The inflammasome provides 'crosstalk' between the innate and adaptive immune systems, and is targeted by the latter.[665]

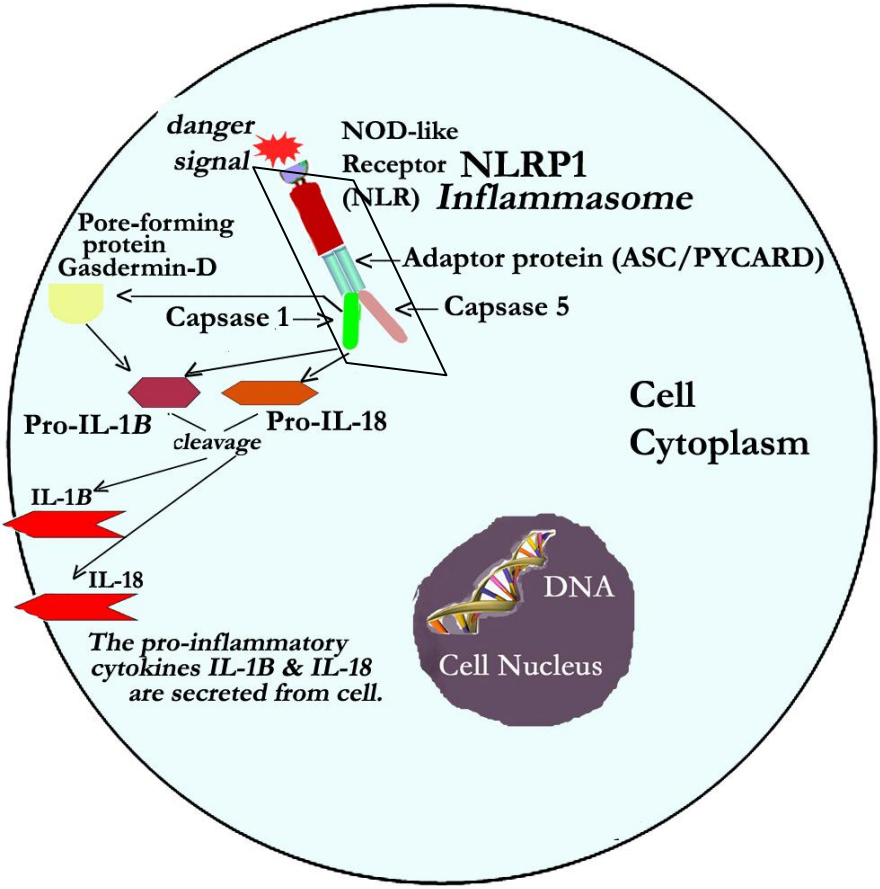
Let's see how this looks, using this simple illustration to get a rudimentary grasp of the structure of the two inflammasomes just described.



[665] Dagenals, Skeldon, and Saleh, "The Inflammasome," 5–6, 8–10.

Let's look at the NLRP1 pathway more closely. It is entirely fitting to focus on this one because its description in 2002 was the first depiction of an inflammasome. To review, the NLR PRR protein NLRP1, with an adaptor protein (ASC) that can link molecules, forms a signaling platform, that then recruits, combines (*dimerizes*) with, and activates a protease named *caspase-1* (CASP-1) via a *caspase activation and recruitment domain* (CARD), which is located at the C-terminus of NLRP1. As a protease, CASP-1 breaks down (cleaves) the inactive precursors to certain proinflammatory cytokines (pro-IL-1β and pro-IL-18); this leads to their active states—*inflammatory cytokines interleukin-1β* (IL-1β) and IL-18. Both IL-1β and IL-18 are proinflammatory cytokines. CASP-1 also cleaves *pore-forming protein Gasdermin-D*, which regulates IL-1β and is noted for inducing cell death associated with inflammation (*pyroptosis*).[666]

The following illustration tries to capture a sense of this pathway. The highlights the signaling platform made of multiple proteins (inflammasome).



[666] Mitchell, Sandstrom, and Vance, "The NLRP1 Inflammasome"; Bell, "Initiation of pro-IL-1β Processing"; and Evavold et al., "The Pore-Forming Protein Gasdermin D."

As noted earlier in this chapter, *interferons* (IFNs) derive that name from their early gained notice of antiviral properties in 'interfering' (hence 'interfere' + particle suffice 'on') with viral replication. They were first identified in the latter part of the 20th century and have been widely studied since. Many cells produce interferons, all of which are *cytokines* (discussed earlier), but not all of the same type. Three different types (I, II, and III) have been described. When cells are stimulated by either a PAMP or a DAMP, PRRs of various types are activated leading to production of interferons that are then secreted to interfere. They might be termed human cell 'resistance fighters' against the invading force.

These types can be broadly distinguished as follows.[667]

	Type I	**Type II**	**Type III** *(Lambda interferons)*
Subtypes	IFN-α (with variants), IFN-β, IFN-δ, IFN-ε, IFN-ζ, IFN-κ, IFN-τ, and IFN-ω	IFN-γ	IFN- λ1, IFN- λ2, IFN- λ3, IFN- λ4 *First classified as interleukins (IL-28a/b and IL-29).*
Receptor	IFNAR complex (IFNAR1 and IFNAR2)	IFNGR complex (IFNGR1 and IFNGR2)	IFNLR complex (IL-28Ra and IL-10R2)
Description	Important in viral defense. Also induced by DAMPs. Mediate cellular immune functions (innate and adaptive systems) for resistance and immunity.	Important in viral defense. Also induced by DAMPs. Involved in allergic response. Defense against intracellular pathogens.	Important in viral defense. Also induced by DAMPs. Active early in infection at epithelial surfaces.

While the different types of interferons play vital roles both in combating virus infection and in modulating the body's antiviral immune response, our focus here is on Type I interferons. These IFNs have become famous for their ability to directly induce an antiviral response, both within infected and surrounding cells. They accomplish this by the increase ('upregulation') of mole-

[667] Table draws on multiple sources, including De Weerd and Nguyen, "The Interferons and Their Receptors"; Dowling and Forero, "Beyond Good and Evil"; Kotenko et al., "Type III Interferons"; Lee and Ashkar, "The Dual Nature of Type I and Type II Interferons"; Zhou et al., "Type III Interferons."

cules that can negatively affect ('antagonize') virus replication. Type I IFNs also activate natural killer (NK) cells so that they perform their immune defense ('effector functions').[668]

Activation of Type-I Interferon responses leads to mediation by way of what is known as the *JAK/STAT pathway* (described below).[669] But let's start with the pattern recognition receptors (PRRs) that might be involved. Immune cells such as macrophages and dendritic cells using TLRs or CLRs may recognize PAMPs and induce cytokines, including Type I IFNs. Inside infected cells, NLRs and RLRs may activate an IFN response.[670] RLRs, for example, recognize viral components (which are PAMPs) and activate *signaling adaptor mitochondrial antiviral signaling* (MAVs), adaptor proteins that then offer a platform to recruit enzymes called *kinases*, which lead to chemical changes involving *interferon regulatory factors* (IRFs) that activate a signaling pathway and lead to IFN synthesis.[671]

PRRs also initiate a chemical process of conversion (*oligomerization*) of MAVs, which leads to transcription factors inducing production both of cytokines and interferons (types I and III). In this manner, mitochondria on host cell membranes provide a platform for signaling complexes that drive production of important immune responses.[672]

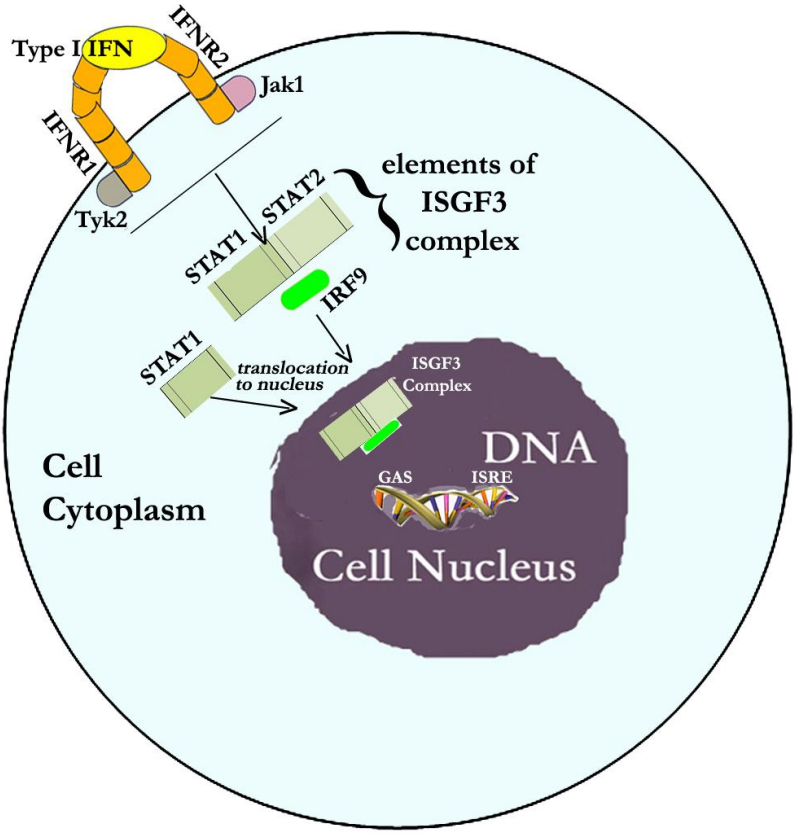
The Type I IFNs all possess a similar structure. Once produced, all of them signal through the *interferon-a/β receptor* (IFNAR) *complex*, a kind of cytokine receptor. This complex is structurally organized like this:

IFNAR1	**IFNAR2**
Four subdomains: *SD 1 SD2 SD3 SD4*	*Two subdomains:* *D 1 D2* *Three isoforms* *IFNAR2a, IFNAR2b, IFNAR2c*

The IFNAR complex is a transmembrane one, with two subunits. The second of these (IFNAR2) has three *isoforms*—i.e., protein structures that vary slightly although their function stays the same.[673] When IFNAR binds to an IFN the result is the IFN is engulfed (*endocytosed*) and it, with the kinases associated with IFNs—*Tyrosine Kinase 2* (Tyk2) protein, and *Janus kinase 1*(Jak1) protein—are activated. IFNAR1 associated with Tyk2 and IFNAR2 with Jak1. Each of these kinases has an N-terminal that recognizes and binds to the near ('proximal') region of the receptor, while its C-terminal regulates its kinase activ-

[668] Lee and Ashkar, "The Dual Nature of Type I and Type II Interferons," 1.
[669] For an overview on all these matters, see Schneider, Chevillotte, and Rice, "Interferon-Stimulated Genes."
[670] Paul, Tang, and Ng, "Interferon Regulatory Factor 9 Structure and Regulation," 1–2. See especially, Swiecki and Colonna, "Type I Interferons," for notes on IFN relation to TLRs and RLRs.
[671] Cui et al., "Mechanisms and Pathways of Innate Immune Activation," 3272.
[672] Wong et al., "Exploiting Connections for Viral Replication," 6.
[673] Zanin et al., "Interferon Receptor Traficking and Signaling," 2.

ity. These associations establish a single signaling unit, which then sets off a classical signaling cascade.[674] This is depicted below (explanation follows).



Kinases, recall, are enzymatic proteins heavily involved in the transmission of signals. The Tyk2 and the Jak1 kinases both belong to the *Janus tyrosine kinase* (JAK) *family*, called 'Janus' because their structure and function bring to mind the ancient two-faced Roman god Janus. Structurally these kinases have two phosphate-transferring domains that closely resemble each other; functionally, one domain exhibits kinase activity while the other negatively regulates that activity. The presence of Tyk2, it might be noted, is the main difference between pathways activated by Type I and Type II IFNs. These two kinases are now activated by the binding of the IFN in the IFNAR complex, which transfer the signal being generated to the cytoplasmic components of the signaling cascade. This is accomplished by changes in the kinases that create so-called 'docking sites' for recruitment of members of the STAT family of proteins.[675]

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[674] Lee and Ashkar, "The Dual Nature of Type I and Type II Interferons," 2, and Zanin et al., "Interferon Receptor Traficking and Signaling," 2. Cf. Smieja et al., "Model-Based Analysis of Interferon-β Induced Signaling Pathway."

[675] Bousoik and Aliabadi, "'Do We Know Jack' about JAK?'" 2–7.

The *signal transducer and activator of transcription* (STAT) *protein family*, just as the name declares, acts both as 'signal transducers' (i.e., players in cell signaling) and as 'transcription factors' (i.e., players in the process of affecting gene expression and subsequent protein synthesis). The activated JAK kinases interact with STAT proteins (STAT1 and STAT2) and this results in creating a protein complex (a *heterodimer*). STAT1 has been reported to exist as a pre-formed *homodimer*—a protein made of two identical polypeptide chains.[676] The STAT1/STAT2 heterodimer is joined by *interferon regulatory factor 9* (IRF9), a transcription factor that mediates the IFN response by regulating the expression of *interferon-stimulated genes* (ISGs).[677]

The STAT1/STAT2 heterodimer plus IRF9 constitute *interferon-stimulated gene factor 3* (ISGF3), a complex that acts as a *transcription factor* (TF), inducing expression of, among others. *interferon regulatory factor 1* (IRF1), a protein-coding gene. Both the STAT1/STAT2 heterodimer and the STAT1 homodimer move ('translocate') into the cell nucleus where they act as TFs.[678]

Below is another illustration about this process, but one that shows what transpires across time in a more vivid manner than that above.

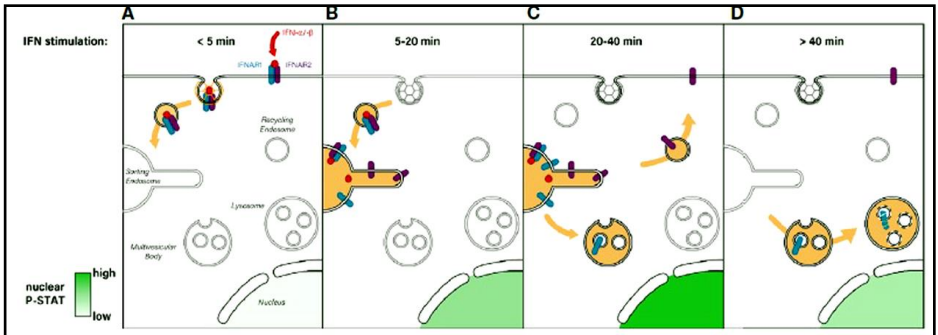

FIGURE 3 | (A) Immediately after type I IFN binding the IFNAR1-IFNAR2 receptor complex is actively endocytosed from the PM and addressed to the endosomal network. (B) Five to 20 min after IFN stimulation, the IFN-IFNAR complex arrives at the sorting endosome where JAK/STAT signaling is triggered, which results in PSTAT nuclear translocation. (C) For the following 20 min, IFNAR signaling is maintained at the endosome where it reaches its maximum (highest P-STAT1 level). The subsequent sorting of IFNAR1 to multivesicular bodies results in signaling termination whereas IFNAR2 is recycled to the PM. (D) After 40 min of IFN stimulation, IFNAR1 is degraded through the lysosomal pathway whereas IFNAR2 is ready for a new round of IFN binding at the PM. Nuclear levels of P-STAT return to prestimulation levels.

PM = plasma membrane (i.e., the cell boundary)

Illustration courtesy of Natacha Zanin, Christine Viaris de Lesegno, Christoph Lamaze, and Cedric M. Blouin, "Interferon Receptor Trafficking and Signaling: Journey to the Crossroads." *Frontiers in Immunology* 11 (Jan., 2021) art. 615603. https://doi.org/10.3389/fimmu.2020.615603. The image, from p. 7, is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/legalcode). No alterations have been made.

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[676] Bousoik and Aliabadi, "'Do We Know Jack' about JAK?'" 7.

[677] Paul, Tang, and Ng, "Interferon Regulatory Factor 9 Structure and Regulation," 1.

[678] See Platanitis et al., "A Molecular Switch from STAT2-IRF9 to ISGF3."

3. Inhibit Virus Regulation

Finally, the host immune system must also make proteins to interfere with viral replication. *Interferons* (IFNs) serve that purpose. This step is a culmination of the three we have been examining: first, pattern recognition receptors (PRRs) sense danger; second, they initiate signaling to send an alert and motivate immune system action; and, third, that action includes production of proteins to interfere with the viral agenda.[679]

A moment ago we saw the culmination of a Type I IFN signaling pathway leads to inducing expression of *interferon stimulated genes* (ISGs).[680] As Sivan Leviyang, whose specialty is modeling viral infection and immune response, points out, "ISGs play a diverse and essential role in the innate immune response, with some acting directly as antiviral effectors while others regulate the innate and adaptive responses."[681] The way they control viral (and bacterial and parasitic) infection is by directly targeting the pathways and functions they need.[682]

In the illustration we focused on *interferon regulatory factor 1* (IRF1), a protein-coding gene. IRF1 is a versatile regulator of various cellular responses. As its name indicates, it regulates transcription of IFN (and IFN-inducible) genes. As we learned in an earlier chapter, 'transcription' means rendering from DNA an RNA copy able to leave the cell nucleus into the cytoplasm, where it directs the building ('synthesis') of proteins—including IFNs.

In the earlier illustration of the classical signaling cascade, and the explanation that followed, it was indicated that the STAT1 homodimer—which is also known as *gamma (γ)–activated factor* (GAF)—activates transcription in response to all types of IFNs. GAFs do so by direct binding to *gamma interferon activation site* (GAS) genes (indicated in the cell nucleus in the illustration). In response to IFN-I, ISGF3 complexes redirect to a distinct group of target genes harboring the *interferon-stimulated response element* (ISRE), the prime mediator of IFN responsiveness in IFN-stimulated genes (ISGs).[683]

In general, cells with a nucleus can, with proper stimulation, produce IFNs. However, some cells—most notably dendritic cells—are expert at it.[684] The IFNs synthesized are then secreted from cell cytoplasm to be detected by other cells. These cells, in turn, are directed to an antiviral stance to resist infection. Inside infected cells IFN actively interferes by restricting the replication cycle.

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[679] For an illustration of the coordination of these steps, see McNab et al., "Type I Interferons in Infectious Disease," 88 (Fig. 1).

[680] Yang and Li, "All about the RNA," 2, succinctly note, "Broadly speaking, an ISG is any gene whose expression is induced by IFN-signaling."

[681] Leviyang, "Interferon Stimulated Binding of ISRE," 1.

[682] Schneider, Chevillotte, and Rice, "Interferon-Stimulated Genes."

[683] Michalska et al., "A Positive Feedback Amplifier Circuit," 2; Leviyang, "Interferon Stimulated Binding of ISRE," 1. On GAS, see Decker, Kovarik, and Meinke, "GAS Elements." On ISRE, see Williams, "Transcriptional Regulation of Interferon-Stimulated Genes."

[684] Kalliolias and Ivashkiv, "Overview of the Biology of Type I Interferons," 2–3.

The Adaptive Immune System

The innate immune system, as we have seen, is not divorced from the adaptive immune system. They constitute a 2-in-1 immune system. But there are enough differences between them to merit discussing them as showing sufficient separation to be distinctive in parts and function. The innate immune system affords the first line of defense; the adaptive immune system quickly reinforces that defense with highly specialized warriors against enemy pathogens.

As we saw earlier, the adaptive immune system provides *specific* responses to challenges to body health and integrity. It is adaptive because it can change; it 'learns' and 'remembers.' Jaydeep Bhat and Dieter Kabelitz, both of the Institute of Immunology at the University of Kiel, Germany, write, "A key feature of the immune system is immunological memory, i.e., the capacity to rapidly respond with increased intensity upon secondary challenge with the same antigen (which forms the basis of vaccination)."[685] The glory of *immunological memory* is that the immune system does not have to keep reinventing the wheel.

Cells of the Adaptive Immune System: B-Lymphocytes (B-Cells)

Earlier in the chapter we saw that the adaptive immune system is *active* and responds to threats via specialized cells. These are called *lymphocytes*. There are two principal types of lymphocytes: B-cells and T-cells.

Discovery and Naming of B-Lymphocytes (B- Cells)

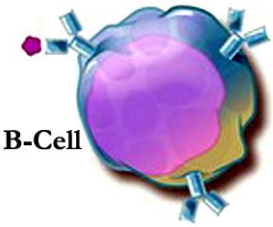

Image courtesy of National Institute of Allergy and Infectious Diseases (NIAID).

B-cells—also known as B-Lymphocytes—are perhaps the most familiar white blood cells. Their name comes from their discovery in the early 1950s and subsequent characterization by researchers in the 1960s, originally based on observation of the *bursa of Fabricus*, an organ in chickens responsible for producing and maturing these cells.[686] Because in adult humans they are made in bone marrow it is easy enough to suppose that is what the 'B' stands for. Although it does not, it still serves as a useful reminder of their place of origin.

Human beings have no *bursa of Fabricus*; instead, B-cells originate in fetal liver or in adult bone marrow. There are two subsets—B1 and B2—but our remarks here concern B2 B-cells.[687] The wonder—and the dilemma—posed by B-cells has been how they are able to produce from a limited set of genes nearly infinite kinds of antigen-specific antibodies. To understand that we must explore the development of B-cells, which we shall do alongside looking more closely at their structure.

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[685] Bhat and Kabelitz, "An Introduction to Immunology and Epigenetics," 1.
[686] For a brief history, see LeBien and Tedder, "B Lymphocytes," 1570–71.
[687] On the distinction, see Graf et al., "BCR-Dependent Lineage Plasticity in Mature B-Cells."

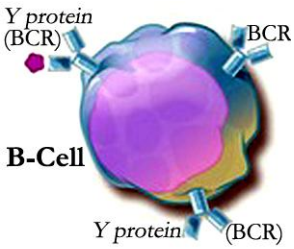
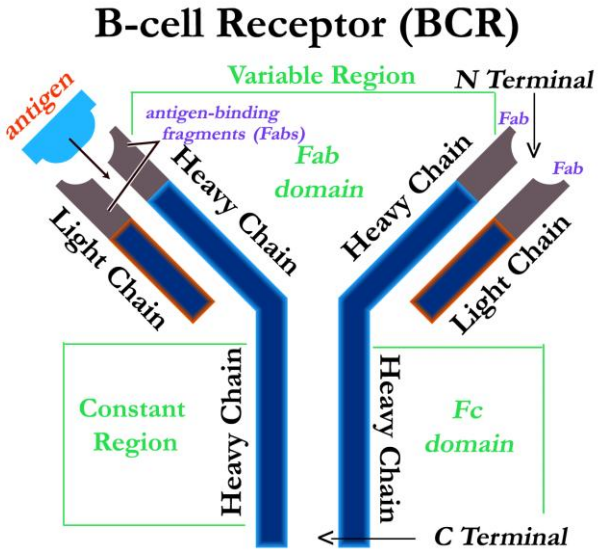


Image courtesy of National Institute of Allergy and Infectious Diseases (NIAID). *(Text added.)*

Let's look again at the image of a B-cell (at left). In bone marrow, via multi-step gene rearrangement, B-cells acquire a *B-cell antigen receptor* (BCR), a Y-shaped transmembrane structure. Like other receptors, the BCR 'receives' contacts from outside the B-cell and 'recognizes' a specific kind of contact—its antigen. The *antibody binding sites* on the BCR are a particular biological challenge. Since B-cells must become specific to one or another of a very large range of possibilities, generating these sites involves a lot of genetic shuffling. This potentially can lead to producing antibody binding sites that erroneously recognize as problems perfectly healthy cells of the body—a process called autoreactivity. Fortunately, there appears to be a process known as *receptor editing* to address this potential issue.[688] There are two types of BCRs (IgD and IgM).

A closer look at this Y-protein BCR may be useful (at right). Like other proteins we have examined, there is a distal N terminal (the region furthest from the cell body) and a proximal C terminal.



Based on an image courtesy of National Human Genome Research Institute (NHGRI) https://www.genome.gov **(All text added.)**

The multitude of very specific antibodies created in the adaptive immune system comes about from gene recombinations that shape the two identical 'heavy' (H) and two identical 'light' (L) chains of amino acids forming the Y-protein. The genes involved in this process are referred to by descriptive labels: *variable* (V), *diversity* (D), and *joining* (J) genes. The process involving these genes is known as *V(D)J recombination*. The heavy chain's V, D, and J genes, and the light chain's V and J genes, undergo permutations in the recombination process to yield a very diverse antibody repertoire. To this *combinatorial diversity* is added *junctional diversity*, an added

[688] See Küppers, "The Life of B Cells according to *JEM*" for a brief overview.

dimension that results from the imprecise joinings at V(D)J junctions when random nucleotides are inserted between the joining genes.[689]

The *fragment antigen-binding* (Fab) domain is the place where binding of the B-cell to its antigen occurs. This is in an area of the receptor known as the *variable region*, so called because this is highly variable across B-cells due to the genetic recombination process. This region is found at the ends of both heavy (V_H) and light chains (V_L). We shall examine both the Fab region and the Fc region of antibodies more closely later.

The *fragment crystallizable* (Fc), found at the base of the Y-protein, is a region that binds to various cell receptors as well as to certain proteins of the *complement system* (or simply *complement*)—a part of the immune system comprised of plasma proteins that can be activated either directly by pathogens or indirectly by pathogen-bound antibodies, but in either instance induce inflammatory responses. The Fc region is found in both chains and accordingly labeled for heavy (C_H) and light (C_L). The Fc domain is found in a place often termed the *constant region* because the molecular make-up of this portion of the protein is relatively constant. This region in different B-cells shows five types of structure, giving rise to the five classes of antibodies we'll discuss a little later.

B-Cell Maturation

B-cell maturation can be divided into the following stages:

- *immature B-cells* formed in bone marrow;
- *transitional B-cells* (differentiated as type 1 (T1) or type 2 (T2)), released from bone marrow; and
- *mature B-cells* (differentiated as 'naive' or 'activated'), formed most famously in the spleen.

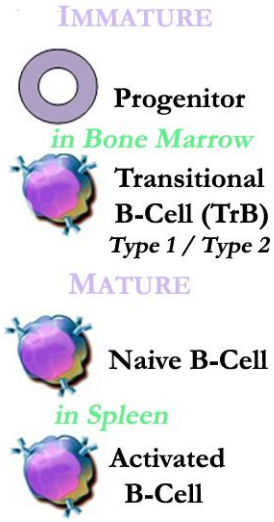
Researcher Florienne Loder and colleagues, at the end of the 20th century, characterized this 'differentiation pathway' to B-cell maturity as "divided into several stages, characterized by differentiation processes, proliferation phases, and control steps"—and governed by a principle they call 'conditional survival.' Through the processes and steps of maturation the B-cell must meet specific requirements to survive and advance. Only a very few do so (e.g., in mice, 1–3% survive from bone marrow to the pool of mature cells).[690]

In bone marrow B-cells develop from a stem cell precursor and undergo steps of differentiation before being released into the blood stream. What eventually results in antibodies being made is, as researchers Tucker LeBien and Thomas Tedder put it, "an error-prone process." Various gene segments are involved with their combinatorial rearrangement. In humans this takes place principally in fetal liver and later in adult bone marrow.[691] Problems during development can lead in an individual to *autoreactivity*—the system treating its own

[689] Collins and Watson, "Immunoglobulin Light Chain Gene Rearrangements," 1–2.
[690] Loder et al., "B Cell Development in the Spleen," 84.
[691] LeBien and Tedder, "B Lymphocytes," 1571.

cells and tissues as though they were dangers, which can result in *autoimmunity*, a system of such responses that underlies autoimmune diseases.

Obviously, the entire process of development is critical to later immune success. Researchers Kathrin Pieper, Bodo Grimbacher, and Hermann Eibel observe in a paper on this matter, "Considering the enormous diversity of antibody specificities, the first challenge of the immune system is to find a balance between variable specificities against pathogens while avoiding auto-reactivity."[692] They point out this requires screening of these cells at various points during their development. But our focus here is about things going right.



The B-cells released into the bloodstream are often called *transitional B-cells* (TrBs) because they have not achieved the final structural form of mature B-cells. The receptor editing referred to above continues through this stage—and after.[693] TrBs are differentiated into Type 1 (T1 B-cells) and Type 2 (T2 B-cells) based on certain structural and functional differences, with T2 B-cells far more responsive to BCR stimulation leading to maturation. T1 B-cells can develop into T2 B-cells.[694] TrBs have been estimated to account for about 4% of normal B-cells in healthy individuals and are found in bone marrow, peripheral and cord blood, and secondary lymphoid tissues (e.g., spleen, lymph nodes, tonsils, and gut-associated lymphoid tissue). Both high and low frequencies of TrBs are associated with various autoimmune diseases.[695] The extinction rate for TrBs is very high: daily some 10–20 million are produced by bone marrow, but only 10% of these survive to reach peripheral tissue—and of them only 10–30% survive to reach the long-lived B-cell pool.[696]

B-cells migrate to the spleen for selection into maturation. Before they become producers of antibodies, these mature B-cells are termed *naive B-cells* (or 'virgin' B-cells). This is because their bodily structure is homogenous—like a generic surface ready to be shaped, they are not yet antigen-specific. These B-cells circulate via the bloodstream and lymphatic system in their circuit through the body, reaching peripheral or secondary lymphoid organs (e.g., spleen, lymph nodes, tonsils, mucosal tissues, and Peyer's patches in the intestines).[697]

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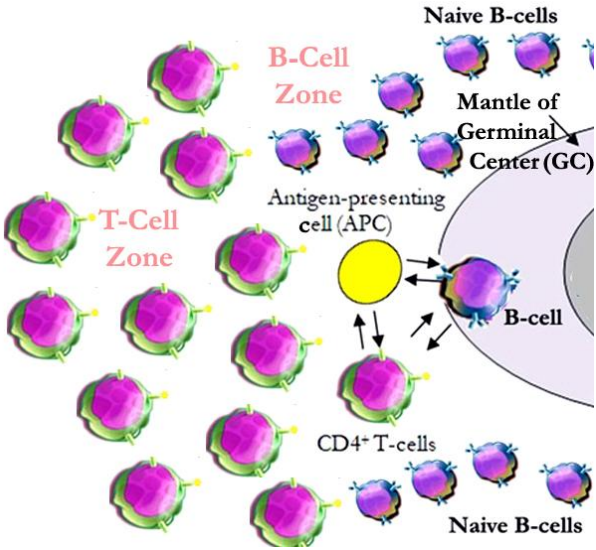
692 Pieper, Grimbacher, and Eibel, "B-Cell Biology and Development," 960.
693 On the whole matter of transitional B-cells, see Martin et al., "Transitional B Cells."
694 Petro et al., "Transitional Type 1 and 2 B Lymphocyte Subsets," 48009.
695 Zhou et al., "Transitional B Cells," 1–2.
696 Petro et al., "Transitional Type 1 and 2 B Lymphocyte Subsets," 48009.
697 Pieper, Grimbacher, and Eibel, "B-Cell Biology and Development," 962. Also see LeBien and Tedder, "B Lymphocytes," 1573.

Some naive B-cells become mature, *activated B-cells*. This is when the B-cell receptor (BCR) described earlier plays a starring role. The fragment antigen-binding (Fab) domain in the variable region of the Y-protein—its 'arms'—recognize an antigen and bind it. Also important is the B-cell's interaction with *helper T-cells* (or, *T-helper cells*). This latter interaction is essential for the proliferation and differentiation that next comes for the B-cell. That process leads us now to a more detailed consideration of this end part of B-cell maturation.

B-Cell Maturation, B-Cell Follicles, and the Germinal Center



In the maturing process, B-cells migrate to T-cell-rich areas termed the *T-cell zone*. Most of them are merely passing through on their way to the *B-cell zone*.[698] But as the illustration at right shows, at the border between these zones an important event takes place—activation of the mature B-cell.

In order for a B-cell to become part of the *germinal center* (GC) *response* (or *GC reaction*)—which we will next examine—it must, through its *B-cell receptor* (BCR), experience a direct, cell-to-cell interaction with another cell—an *antigen-presenting cell* (APC), which is one that presents for recognition a substance that provokes an antibody response, called an *antigen* (Ag). APCs are cells such as *follicular dendritic cell* (FDC), macrophage, or dendritic cell. This event is known as *recognition of a cognate antigen*.

As we have noted earlier, the B-cell also interacts with helper T-cells (T_h-cells) known as *CD4-positive T-cells* ($CD4^+$ T-cells, or sometimes just called 'T4 cells'). A *cluster of differentiation 4* (CD4) *glycoprotein* serves as a co-receptor on the T_h-cell—hence its name. The $CD4^+$ T-cells recognize certain molecules presented on these APCs. The $CD4^+$ T-cells provide the B-cells with survival and co-stimulatory signals. After this, the activated B-cell moves to the center of the primary B-cell follicle to help seed the GC response (discussed further below).[699] Any antigen that requires the presence of T_h-cells is known as a *T-cell dependent antigen* (Td-Ag). The encounter makes the B-cell an a*ctivated B-cell*.

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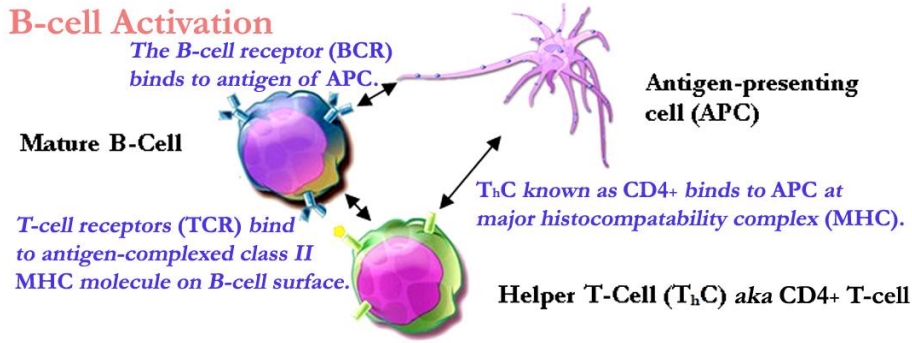
[698] Janeway et al., *Immunobiology*, 349.

[699] Stebegg et al., "Regulation of the Germinal Center Response," 2. Also see Klein and Dalla-Favera, "Germinal Centres," 22, following MacLennan, "Germinal Centers."

Let's look closer. As the illustration below shows, activation of a B-cell involves a set of interactions. The B-cell through its receptor (BCR) takes up antigen, internalizes it, and presents it to helper T-cells.[700] It thus acts as a *professional APC* for the CD4+ T-cell by expressing cell-surface major histocompatibility complex class II (MHCII) molecules with bound peptide (the ligand of the α/β T-cell receptor).[701] The CD4+ T-cell also keys on the *peptide major histocompatibility complex class II* (pMHCII) presented by an APC (here a dendritic cell (DC)), which activates the T_h-cell.[702] This binding process involves both the T-cell receptor (TCR) and the CD4 protein acting as co-receptor. The choice here of a DC as the APC recognizes that among APCs dendritic cells are considered especially important because of their enhanced ability to stimulate naive T-cells.[703] This complex of interactions activates the mature B-cell.



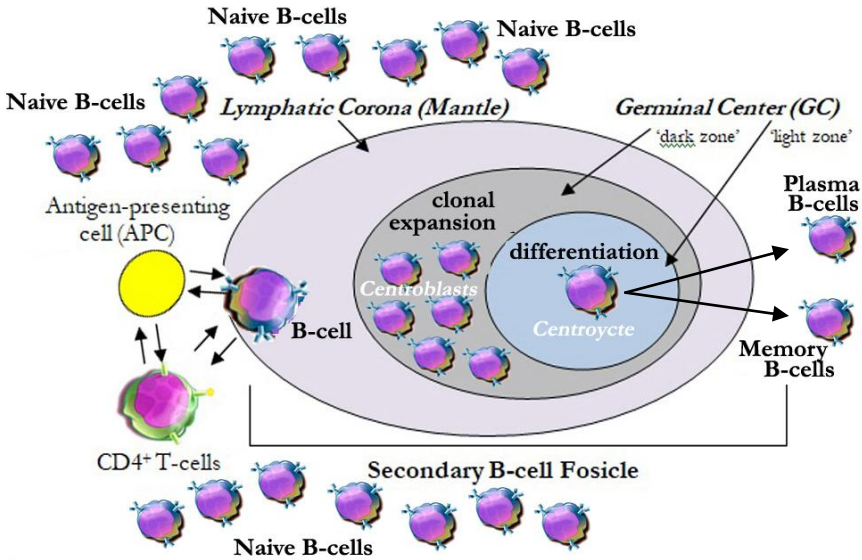
Based principally on research with rodents (*murine* models), the B-cell activation sequence and sequelae look like this:

Time Sequence	Event
	Naive B-cell is presented an antigen by an antigen-presenting cell (APC)
within 48 hours	At T-cell border both some B-cells and some T-cells respond by maturing directly to plasmablasts (PBs). Some B-cells migrate to extrafollicular regions and begin PB differentiation.
within 4–6 days	Peak of antibody (Ab) secretion and proliferation. Some B-cells (and some T-cells) migrate into B-cell follicles (FO Bs), where they continue proliferation
within 5 days	Germinal center (GC) are initiated and develop.[704]

[700] For details, see Avalos and Ploegh, "Early BCR Events and Antigen Capture."
[701] Adler et al., "The Other Function: Class II-Restricted Antigen Presentation by B Cells," 2.
[702] For more on this matter, see Wieczorek et al., "Major Histocompatability Complex."
[703] Luckheeram et al., "CD4+ T Cells," 2.
[704] This table's contents derived from a number of sources but was first inspired by the discussion in Elsner and Shlomchik, "Germinal Center and Extrafollicular B Cell Responses," 1137.

In the B-cell zone, B-cells home in on *primary B-cell follicles* (or, *follicular B-cells* (FO Bs)). These are a complex network comprised of not only B-cells but *follicular dendritic cells* (FDCs) and others. The FDCs secrete a chemokine (*B lymphocyte chemokine* (BLC)) that attracts both naive and mature, activated B-cells.[705] A *secondary B-cell follicle* develops when an antigen is encountered. About one week after such exposure a *germinal center* (GC)—a specialized microstructure—forms in the midst of naive B-cells. This structure pushes aside the naive B-cells.[706]

The Germinal Center (GC) mantle crowds out the naive B-cells; clonal expansion and differentiation occur in GC.



The illustration, of course, is a static, frozen picture. In reality the situation is dynamic. As researchers Gabriel Victora and Michel Nussenzweig remark in a paper on the germinal center, "B cells not only move constantly throughout the entire GC, but also engage in short, dynamic interactions with both T cells and antigen."[707]

Not only is the situation dynamic, but it is also more complex than the above suggests. The so-called 'canonical GC response,' which we are focused upon, is commonly preceded by a short phase of *extrafollicular* (EF) proliferation and differentiation. Moreover, noncanonical reactions typically have prolonged reactions at EF sites.[708] But—remember—we are staying in the shallow end of the pool and shall content ourselves with a more limited focus.

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[705] Janeway et al., *Immunobiology*, 350 and Sagaert, Sprangers, and De Wolf-Peeters, "The Dynamics of the B Follicle," 1378.
[706] Victora and Nussenzweig, "Germinal Centers," 431.
[707] Victora and Nussenzweig, "Germinal Centers," 431.
[708] Elsner and Shlomchik, "Germinal Center and Extrafollicular B Cell Responses," 1136.

The GC classically is represented as having two zones—a 'dark' and a 'light' one—with a mantle known as the *lymphocytic corona*. One zone is termed 'dark' because under light microscopy it appears so as a result of B-cells with a high *nucleocytoplasmic ratio* (N:C ratio)—the proportion of volume of the nucleus compared to the surrounding cell cytoplasm; as cells mature the size of the nucleus relative to the cytoplasm decreases. The B-cells in the dark zone (DZ) acquired the name *centroblasts* (a 'central' (*centro-*) but 'immature' (*-blast*) B-cell)—large and quick to proliferate, but still lacking the differentiation acquired in the light zone (LZ). The latter looks lighter because less crowded with B-cells—now termed c*entrocytes*—that interact with the FDCs mentioned earlier and *T-follicular helper* (Tfh) cells. In more recent years the supposed distinctions between centroblasts and centrocytes has been challenged and eroded, but the terminology remains convenient to denote if the B-cell is in the DZ or the LZ.[709]

While most of the lymphocytes in the GC are activated B-cells (which are larger than naive B-cells), about 10% are antigen-specific T-cells. These GC B-cells are among the fastest dividing cells found in mammals—a distinct advantage in immune defense where a rapidity of response is highly desirable. These cells have a cell-cycle time estimated at 6–12 hours. As mentioned a moment ago, in the light zone selected B-cells encounter and interact with other immune system cells such as FDCs and Tfh cells.[710]

In the LZ some B-cells express newly mutated BCRs, capture antigen presented by FDCs, and then internalize it for presentation to helper T-cells. (This is the same process described earlier concerning the activation of a mature B-cell.) This activity concluded, the B-cell is now subject to a selection decision that determines whether it is recycled to the DZ for further *affinity maturation* (the mutating to achieve higher affinity with a specific antigen), or chosen for differentiation and exit from the GC as either a long-lived plasma cell or a memory B-cell (both examined a little later).[711]

A third illustration (next page) can now better show how the cells and events we have been describing come together in a dynamic GC response. In the GC's dark zone, B-cells undergo modifications in which mutations accumulate in its antibody V-regions (both heavy and light chains)—a process known as *somatic hypermutation* (SHM). In the light zone B-cells also experience *affinity maturation*, a process favoring survival of those B-cells with the highest affinity for the antigen for which they are specific. B-cells also experience *isotope switching*, a process facilitating them expressing effector functions as antibodies.[712]

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[709] Sagaert, Sprangers, and De Wolf-Peeters, "The Dynamics of the B Follicle," 1378, Stebegg et al., "Regulation of the Germinal Center Response," 2, and Victora and Nussenzweig, "Germinal Centers," 431–33. Cf. Camacho, Kosco-Vilbois, and Berek, "The Dynamic Structure of the Germinal Center."

[710] Victora and Nussenzweig, "Germinal Centers," 431, and Janeway et al., *Immunobiology*, 350.

[711] Inoue et al., "Exit from Germinal Center," 1.

[712] Janeway et al., *Immunobiology*, 350, and Nakagawa and Calado, "Positive Selection in the Light Zone," 1.

Let's now zoom in even closer with this more detailed illustration.

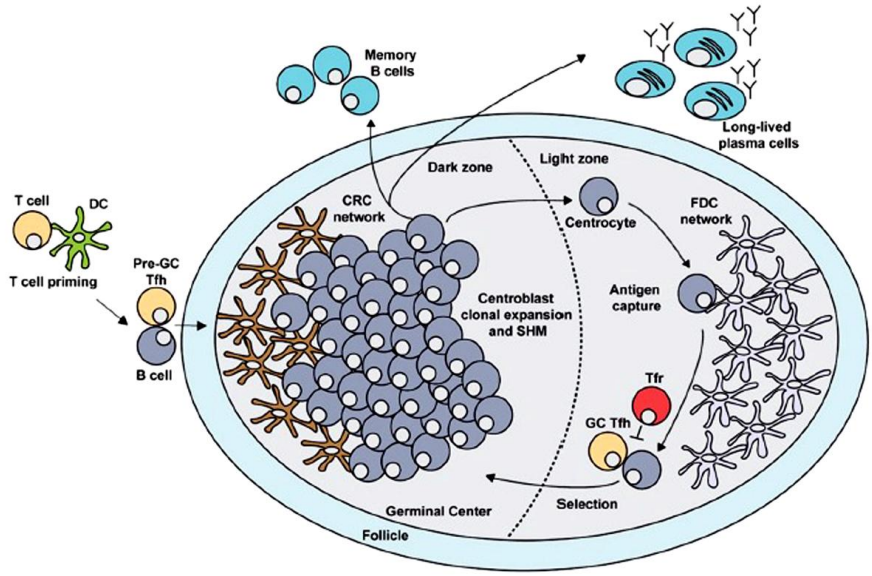

FIGURE 1 | The germinal center (GC) response. The GC is a specialized microenvironment formed within the B cell follicles of secondary lymphoid tissues upon infection or immunization. The GC is divided into two distinct compartments. The dark zone (DZ) that contains a network of CXCL12-producing reticular cells (CRCs) and is the site of GC B cell proliferation and somatic hypermutation (SHM). Centroblasts then follow a CXCL13 gradient to enter the light zone (LZ) as centrocytes through their expression of CXCR5. In the LZ, centrocytes capture antigen presented on follicular dendritic cells (FDCs) which they internalize, process and subsequently present to T follicular helper (Tfh) cells in order to undergo selection. This process is regulated by T follicular regulatory (Tfr) cells which are also present in the LZ. Upon receiving survival signals from Tfh cells, centrocytes re-enter the DZ for further rounds of proliferation and SHM after which they exit the GC as memory B cells or high-affinity antibody-secreting plasma cells.

Illustration courtesy of Marissa Stebegg, Saumya D. Kumar, Alyssa Silva-Cayetano, Valter R. Fonesca, Michelle A. Linterman, and Luis Graca, "Regulation of the Germinal Center Response." *Frontiers in Immunology* 9 (Oct., 2018) art. 2469. doi: 10.3389/fimmu.2018.02469. The image, from p. 3, is made available by Creative Commons Attribution (CC BY) License (https:// creativecommons.org/licenses /by/4.0/legalcode). No alterations have been made.

This final illustration may push to our tip-toes in the pool, so let's back up and return to an earlier point—the end products of this GC process, which can be seen at the top of the above picture. As we have seen, some B-cells differentiate into either memory B-cells (MBCs) or long-lived plasma cells (LLPCs). Both important players in the adaptive immune system require a closer look.

Long-lived plasma cells (LLPCs) are antibody-secreting cells; bone marrow plasma cells (BMPCs) are the principal source of antibodies. Plasma cells are terrifically prolific, able to secrete antibodies at an exceptionally rapid rate (~10^3/second). LLPCs are distinguished from *short-lived plasma cells* (SLPCs, aka *plasmablasts*), rapidly repopulating (i.e., 'proliferating') cells which have a life span of just 3–5 days. LLPCs, which do not proliferate, can survive several months or even much longer. These two types of plasma cells differ further in that SLPCs predominately originate at extrafollicular (EF) sites in secondary lymphoid organs expressing low-affinity IgM antibodies. LLPCs, though, are mainly formed by GC reaction as we have seen.[713] LLPCs emerge from the light zone (LZ) of the germinal center (GC). Like those B-cells destined to be recycled and those selected to be memory B-cells, cells destined to be LLPCs appear to express a protein, *interferon regulatory factor 4* (IRF4), which is a critical transcription factor for differentiation into plasma cells.[714]

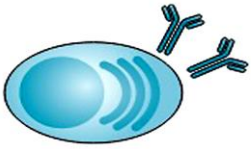

Image courtesy of National Institute of Allergy and Infectious Diseases (see Akkaya, Kwak, and Pierce, "B Cell Memory" article).

Once differentiated as such, LLPCs reside in locations favorable to their survival—what have been termed 'pro-survival niches.' These are found in bone marrow—the place most often associated with LLPCs—but also in secondary lymphoid organs, mucosa, and places where inflammation is happening. In fact, in humans about 80% of LLPCs are found in *gut-associated lymphoid tissue* (GALT). GALT LLPCs produce primarily IgA. Bone marrow (BM) LLPCs are noted for producing IgG, but they also produce IgA (~40%). In fact, BM seems to be a place where LLPCs secreting IgM and IgE, too.[715]

While both SLPCs and LLPCs play vital roles in immune response by producing antibodies, it has been suggested that evidence such as that discussed above leads to distinguishing different primary roles. Thus immunologists Shivana Lightman, Adam Utley, and Kelvin Lee propose that the primary role of SLPCs is protection against frequent (and less severe) endemic infections sustained by recurrent and antigen-dependent B cell reactivation, whereas LLPCs provide sustained protective immunity against infrequent (but more severe) epidemic infections in an antigen-independent fashion.[716] LLPCs immediately rush to the front lines to provide protection against the enemy pathogen. Memory B-cells (MBCs) are ready in reserve and only engage if the BMPC-derived antibodies do not fully neutralize the danger.[717]

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[713] Khodadadi et al., "The Maintenance of Memory Plasma Cells," 1–2.
[714] Inoue et al., "Exit from Germinal Center," 1; Palm and Henry, "Remembrance of Things," 1.
[715] Lightman, Utley, and Lee, "Survival of Long-Lived Plasma Cells (LLPC)," 2.
[716] Lightman, Utley, and Lee, "Survival of Long-Lived Plasma Cells (LLPC)," 2.
[717] Kim et al., "Germinal Centre-Driven Maturation of B Cell Response," 3.

Image courtesy of National Institute of Allergy and Infectious Diseases (see Akkaya, Kwak, and Pierce, "B Cell Memory" article).

Memory B-cells (MBCs) are key to *immunological memory*—the body's ability to remember past dangers, recognize them as they reoccur, and elicit a rapid and effective response. Not all B-cells become MBCs, of course. Those that are most likely to become so express protein markers, and this subset of B-cells has become called by some researchers *pro-memory B-cells*. The *BTB domain and CNC homolog 2* (BACH2) protein, which regulates the process by which select activated B-cells become MBCs has been specially noted.[718] However, other factors also have been associated with MBC generation: proteins *activated B-cell factor-1* (ABF-1), *Kruppel-like factor 2* (KLF2), *signal transducer and activator of transcription 5* (STAT5), and *zinc finger and BTB domain containing 32* (ZBTB32).[719] MBCs, like the antibody-secreting cells (ASCs)—notably the SLPCs and LLPCs just discussed—are the product of activation by an antigen, typically alongside interaction with cognate helper T-cells.[720]

MBCs emerge from the LZ of the GC ready to elicit rapid immune response upon reexposure to a specific antigen (a 'secondary antigen challenge'). They work in tandem with LLPCs to provide *protective memory*. First, LLPCs secrete circulating antibodies. If these are not enough, MBCs engage. This necessitates MBCs be stationed strategically, such as in the primary lymphoid bone marrow and secondary lymphoid organs like the spleen, the lymph nodes (in the subcapsular sinus (SCS)), tonsils, Peyer's patches in the small intestine, and the gastro-intestinal tract; they also circulate in blood. Some MBCs reside at sites of infection, serving as *tissue-resident MBCs* with especially rapid activation.[721]

Though MBCs do not themselves secrete antibodies, when activated, MBCs proliferate rapidly and can differentiate into plasma cells. They also can interact with $CD169^+$ macrophages to form new GCs.[722] MBCs differ by antibody type, each with their own distinct functions. IgG^+ MBCs preferentially differentiate into LLPCs. On the other hand, IgM^+ MBCs for the most part enter the GC reaction. IgE MBCs, if present at all, are found only as a small population.[723]

Though it has been said before, immunobiologist Whitney Purtha and colleagues succinctly sum the respective roles of LLPCs and MBCs in terms worth learning: "Long-lived plasma cells (LLPCs) constitutively secrete antibody to neutralize antigen immediately upon reinfection, whereas memory B cells (MBCs) produce antibody only upon restimulation by specific antigen."[724]

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[718] Inoue et al., "Exit from Germinal Center," esp. 10–13.
[719] Palm and Henry, "Remembrance of Things," 2.
[720] Palm and Henry, "Remembrance of Things," 1.
[721] Palm and Henry, "Remembrance of Things," 4.
[722] Palm and Henry, "Remembrance of Things," 4.
[723] Kurosaki, Kometani, and Ise, "Memory B Cells."
[724] Purtha et al., "Memory B Cells, But Not Long-Lived Plasma Cells," 2599. Also see Akkaya, Kwak, and Pierce, "B Cell Memory: Building Two Walls of Protection against Pathogens."

The Adaptive Immune System: B-Cell Produced Antibodies

B-cells are noted for their production of key players in the immune defense system: proteins called antibodies, more formally known as immunoglobulins. *Antibody* is a name descriptive of function—antibodies are a group of cells that fight against invaders. On the other hand, the term *immunoglobulins*—from 'immune' (*immuno-*) 'minute blood cells' (*-globulin*)—is a biochemical name for the group of simple proteins that dissolve in water diluted by salt. While both terms name the same entities, immunoglobulins is a broader term.

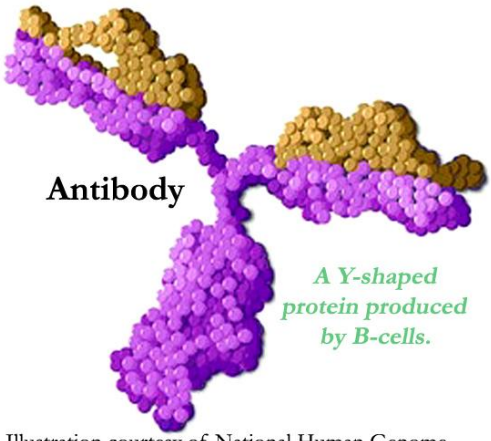

Illustration courtesy of National Human Genome Research Institute (https://www.niaid.nih.gov) *Text added.*

Antibodies are the most famous warriors of the human immune system. In structure they are Y-shaped proteins (see illustration at left). This should immediately call to mind the form of the B-cell receptor (BCR), which we looked at earlier.

Antibodies Compared to BCR

The BCR is a transmembrane molecule rooted in the B-cell and extending to recognize antigens presented to it. An antibody, however, lacks a transmembrane domain (TM); in technical terms it is not 'membrane-bound.' Here are differences:

Membrane-Bound Ig	Soluble, Secreted Ig
This functions as a BCR, controlling maturation, activation, and differentiation of B cells.	Known as antibody (Ab), this functions as adaptive immune response in surveillance, recognition of a pathogen, and immune reaction.[725]

Obviously, membrane-bound Igs do not detach; they are not 'secreted.' But antibodies are. These are soluble and secreted into the bloodstream, where they are among the most abundant protein components in blood.[726]

Another important difference, as indicated above, lies in function. As we have seen, the BCR binds with a specific antigen and interacts with a helper T-cell to become a mature, activated B-cell. Antibodies also bind to specific antigen, but for a different purpose—containment and destruction of a pathogen.

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[725] Iype et al., "Differences in Self-Recognition," 1417; Mak and Saunders, *Immune Response*, 105.
[726] Alberts et al., *Molecular Biology of the Cell*, 1315.

Finally, while there are two famous types of BCRs—IgD and IgM—there are actually five types (*isotopes*) of immunoglobulins (Ig), and each is usually referred to by the abbreviation Ig followed by a letter for the type. These are:

Type	% as antibodies (Abs) in Blood Serum	
IgG	about 75–80% of antibodies in blood serum.	
IgA	about 13–15% of antibodies in blood serum.	
IgM	about 5% of antibodies in blood serum.	**BCR**
IgD	about 0.2% of antibodies in blood serum.	**types**
IgE	only a trace amount of antibodies in blood serum.	

Let's discuss the BCR types of Ig first. Mature B-cells are noted for expressing two BCR isotopes—IgD and IgM. The BCR is made of *membrane immunoglobulin* (mIg), with IgD possessing a structure of four immunoglobulin domains in its heavy (H) chain and IgM having five such domains. Each type of BCR also has a short intracellular domain of three amino acids (lysine-valine-lysine).[727] At first, B-cells express only IgM BCR. Typically, IgD expression begins in transitional B-cells. Both IgD and IgM BCR are found in mature B-cells. In principle, though, all five isotypes can be spliced as the membrane-associated mIg form, thereby presenting as BCR on the B-cell surface. In actual practice, however, the BCR isotopes are not equally used.[728]

Antibody Classes: General Comparison

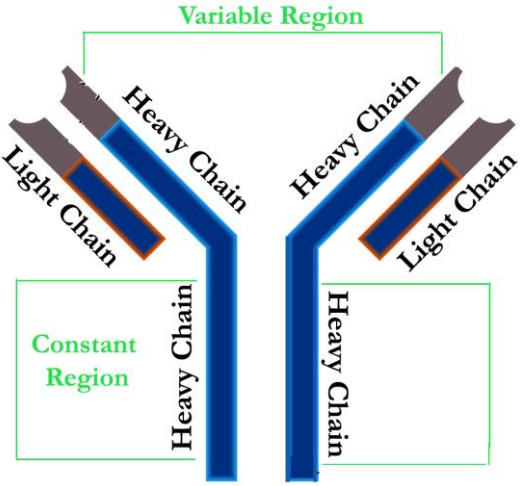
Our focus is on soluble and secreted Igs, called *antibody-secreting cells* (ASCs). They include both short-lived and long-lived plasma cells. They differ among themselves structurally in the makeup of their H chains (which provide their isotope designations), and also in distribution. Some differences are:

Class (Isotope)	**Heavy Chain Composition**	**Ig Subclasses** (9 'subclasses' when adding IgM, IgD, IgE)	**Distribution**
IgG	*gamma* (γ) chain	IgG1, IgG2, IgG3, IgG4	intracellular; extracellular
IgA	*alpha* (α) chain	IgA1, IgA2	intravascular; secretions
IgM	*mu* (μ) chain	--	principally intravascular
IgD	*delta* (δ) chain	--	surface of lymphocytes
IgE	*epsilon* (ε) chain	--	basophils; mast cells (saliva and nasal secretions)

[727] Treanor, "B-Cell Receptor: From Resting State to Active," 21.
[728] Maity et al., "Isotope Specific Assembly of B Cell Antigen Receptors," 2–3; cf. Friess, Pluhackova, and Böckmann, "Structural Model of the mIgM B-Cell Receptor," 1.

To put the above a little bit differently, let's reproduce an earlier illustration to highlight some differences in structure. In the table above we saw one important area of difference among the antibodies lies in their heavy (H) chains. Another area of difference is in distinct units ('domains') within the variable and constant regions. The variable (V) region relates to the antigen-binding site; the constant (C) region, with its greater fixity, determines the isotope.

Three of these antibody types (IgA, IgD, and IgG) have 3 C domains and 1 V domain. On the other hand, the remaining two types (IgE and IgM) have 4 C domains and 1 V domain. One member of each of these two groups (IgA and IgM) have an additional *joining* (J) *chain*—a polypeptide chain encoded by the IGJ gene that not only promotes linkages but also regulates formation such that these two antibodies form a dimer (IgA) and a pentamer (IgM).[729] We'll see what these differences look like structurally, but first we need to focus on the heavy chains.



Based on an image courtesy of National Human Genome Research Institute (NHGRI) https://www.genome.gov **(All text added.)**

Mature B-cells begin by expressing principally IgM, but also IgD on their surface. Antigen activation of a B-cell may lead to another antibody class—an event called *class switching*—by which the production of antibody may become one of the other classes (e.g., IgA, IgG, or IgE) as needed. This happens, as described above, by a DNA recombination event—*class switch recombination* (CSR)—that replaces the *mu* (μ) and *delta* (δ) *heavy chain constant* (C_H) *regions* of the originally expressed Ig with *gamma* (γ), *epsilon* (ε) or *alpha* (α) C_H regions. The purpose of such switching is to enhance the body's ability to eliminate the pathogen that induced the antibody response.[730] So the first responders of the adaptive system response are IgM-expressing B-cells. But in subsequent invasions by the pathogen memory B-cells may produce other Ig types against that pathogen. Thus, for example, in one place the pathogen might be met by IgA (e.g., in external excretions), but in another place by IgG (e.g., in blood). With each subsequent exposure to the antigen the adaptive response becomes more specific and does so using this ability to switch classes of antibodies.[731]

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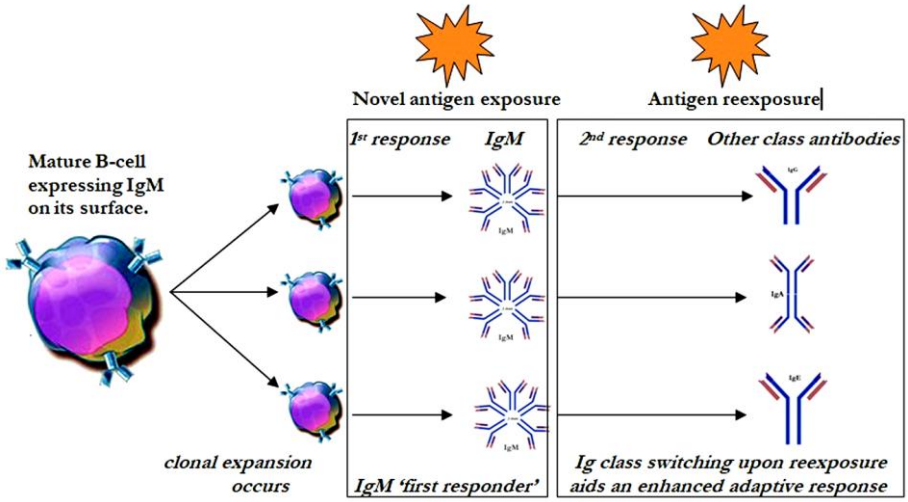
[729] Chiu et al., "Antibody Structure and Function," 2.
[730] Stavneser and Schrader, "Ig Heavy Chain Class Switch Recombination," 5370.
[731] Mak and Saunders, *The Immune Response*, 104–05.

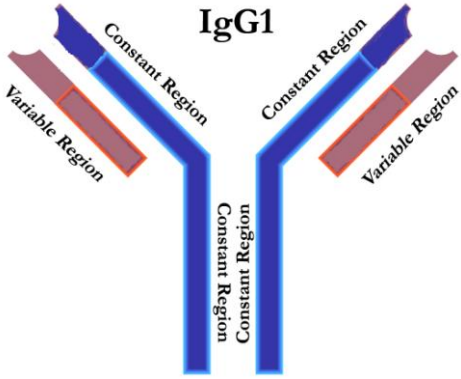
Illustrated simply, the adaptive response mounted by B-cells looks like this:



Switching changes the antibody *effector function*—the 'effect' produced by some specific 'function' done by the antibody. Most effector functions are induced via the fragment crystallizable (Fc) domain of the constant (C) region of the antibody. This Fc region can interact with specialized Fc receptors and with *complement* proteins (small proteins that enhance—'complement'—antibody actions). Among others, effector functions include such acts as interfering with pathogens binding to healthy cells or facilitating ingestion of a pathogen. [732]

Antibody Types, 1: IgG

The most common antibody (Ab) in blood serum is *immunoglobulin gamma heavy chain* (IgG), accounting for three-quarters or more of antibodies found in serum of healthy adults under normal conditions. IgG is the principal antibody found not only in blood serum, but in lymph fluid and cerebrospinal fluid; it is also able to cross the placenta and bears chief responsibility for immune protection prenatally and after birth for the first few months through breast milk.[733] It is also the longest-lived



[732] Van Erp et al., "Fc-Mediated Antbody Effector Functions," 2.
[733] Pierzynowska et al., "Maternal Immunoglobulins in Infants."

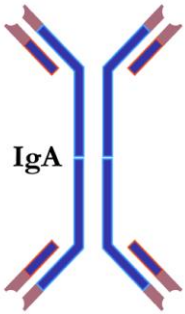
antibody, with a *half-life* (i.e., the time required to eliminate naturally half the substance) of about three weeks (~23 days) for IgG1, IgG2, and IgG4 (with IgG3 only 2–6 days).[734]

As noted in an above table, IgG has four subclasses. The most abundant is the one pictured (IgG1), with the others becoming less common as subclass number goes up (thus IgG4 is least common). These subclasses differ in their constant region, where binding to *fragment crystallizable gamma receptors* (FcγRs) and *complement component 1q* (C1q) occurs.[735] Substantively, what these differences mean are somewhat variable effector functions (i.e., the activities carried out in response to stimulation) for the subclasses.

IgG is able to neutralize pathogens by interfering with their ability to interact with host cells. They do this by binding to the pathogen surface at proteins the antibody recognizes. Among other things, IgG assists phagocytes—cells that engulf and absorb pathogens—by enabling them to recognize dangerous substances, including viruses. IgG bound to an antigen creates an *immune complex* (IC). An IgG IC 'complements'—i.e., enhances—the immune response. This is a normal part of immune response but can be a danger to the body when it goes out of control.[736]

Antibody Types, 2: IgA

IgG is five times more common than *immunoglobulin alpha heavy chain* (IgA), the second most common antibody found in blood serum. IgA, as noted earlier, has 3 constant domains and 1 variable domain. It has two subclasses (IgA1 and IgA2), which vary by just 13 additional amino acids contained in the hinge region of IgA1.[737] Also as noted above, IgA has an additional joining (J) chain, which renders the structure of a *dimer*—two identical parts joined together—a structure we have met with elsewhere in this volume.[738] Its half-life is considerably less than IgG—estimated to be only 4–6 days,[739] a factor limiting its appeal therapeutically.[740] IgA reacts with a variety of receptors on host cells (*fragment crys-*



[734] Saxena and Wu, "Advances in Therapeutic Fc Engineering," 2; compare to immunoglobulin for intravenous use (IGIV) where total Ig half-life of 25.8 days was reported in one study (with range from 29.7 days for IgG1 to 15.7 days for IgG3—see Mankarious et al., "The Half-Lives of IgG Subclasses."

[735] Vidarsson, Dekkers, and Rispens, "IgC Subclasses and Allotypes."

[736] For details, see Karsten and Köhl, "The Immunoglobulin, IgG Fc Receptor and Complement Triangle in Autoimmune Diseases."

[737] Sterlin and Gorochov, "When Therapeutic IgA Antibodies Might Come of Age," 10.

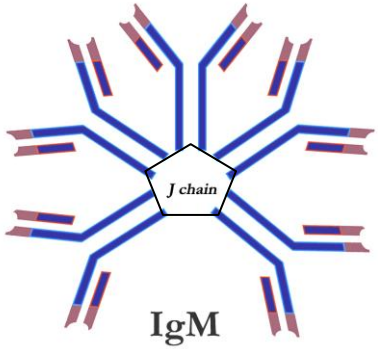
[738] Both sub-types can also have monomer forms; see De Sousa-Pereira and Woolf, "IgA: Structure, Function, and Developability," 3 (Fig. 2).

[739] De Sousa-Pereira and Woolf, "IgA: Structure, Function, and Developability," 15.

[740] Sterlin and Gorochov, "When Therapeutic IgA Antibodies Might Come of Age," 13.

tallizable alpha receptor I (FcαRI), *polymeric immunoglobulin receptor* (pIgR), and *fragment crystallizable alpha/mu receptor* (Fcα/μR, also known as CD351)).[741] IgA neutralizes or blocks activity by a range of viruses, bacteria, and protozoa, and prevents their attachment to host cells.[742]

Antibody Types, 3: IgM



Researchers Siqi Gong and Ruth Ruprecht describe *immunoglobulin mu heavy chain* (IgM) as "the first responder to foreign invaders—including viral pathogens that cause major pandemics. It is the only antibody class that exists in all vertebrate animals."[743] When a new ('novel') pathogen is encountered that the innate system does not quately counter it is IgM antibodies that are predominantly produced.

IgM accounts for about 5% of the antibodies found in blood serum. It has 4 constant domains and 1 variable domain. Like IgA, IgM has an additional joining (J) chain, which renders the structure of a *pentamer* (pictured)—five sub-units, that contain 10 antigen binding sites and the joining (J) chain.[744] But IgM can take variable forms. For example, it is the B-cell antigen receptor (BCR) as a *monomer*. But when secreted—our key interest here—IgM is predominantly pentameric and contains the joining (J) chain. This chain allows IgM to be transported across the thin tissue barrier in the mucus membranes lining many organs (*mucosal epithelia*) through binding with the *polymeric immunoglobulin receptor* (pIgR), an interaction that leads to the formation of secretory IgM.[745] Its specific *fragment crystallizable* (Fc) receptor is FcμR[746] and through its interaction with Fc receptors it regulates the immune system.[747]

IgM is found mainly in blood serum and lymph fluid. In blood serum, where it has a half-life of 5–6 days, a 5–10 day lag transpires before the antibodies appear in blood (with peak concentrations at 21 days).[748] However, IgM can be secreted across mucosal surfaces and plays a key role in mucosal defense. IgM production is also found with *mucosa-associated lymphoid tissues* (MALT)—

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741 Woof and Russell, "Structure and Function Relationships in IgA," 590–91. On FcαRI, see Aleyd, Heineke, and Van Egmond, "The Era of Immunoglobulin A Fc Receptor FcαRI." On Fcα/μR, see Yang et al., "The Three Complementarity-Determining Region-Like Loops."
742 De Sousa-Pereira and Woolf, "IgA: Structure, Function, and Developability," 9.
743 Gong and Ruprecht, "Immunoglobulin M," 1.
744 Keyt et al., "Structure, Function, and Therapeutic Use of IgM Antibodies," 1.
745 Gong and Ruprecht, "Immunoglobulin M," 1.
746 On FcμR, see Liu et al., "Role of the IgM Fc Receptor in Immunity and Tolerance."
747 Gong and Ruprecht, "Immunoglobulin M," 3.
748 Flaherty, *Immunology for Pharmacists*, 72, 74.

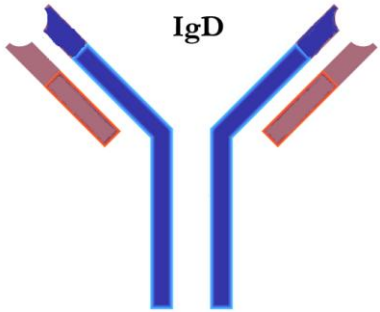
secondary lymphoid tissues that produce both B-cells and T-cells—these being associated with multiple organ systems, including the gastrointestinal and respiratory tracts.[749] IgM plasma cells (PCs)—effector functioning antibodies—account for 10–20% of all PCs found in the human gut.[750]

Because it is produced by both B1 and B2 B-cells, a distinction is made between 'natural IgM' produced mainly by B1 cells, and 'pathogen-induced IgM,' made by both B1 and B2 cells.[751] The former represent the majority of secreted IgM antibodies found in normal serum.[752] Our focus here is on the latter.

IgM exhibits potent *complement dependent cytotoxicity* (CDC) enabled by its pentameric (or sometimes hexameric) structure.[753] IgM possesses high *avidity*—a measure of its accumulated strength across interactions with its ligands—which enables it to bind antigen effectively, even with relatively low affinity binding sites. Moreover, IgM as an antibody (antigen-specific IgM) plays dual roles: one is direct for antigen destruction and the other regulatory, but both involve the *complement system*. This system often is referred to simply as *complement* and involves plasma proteins that react with one another to coat pathogenic particles (a process termed *opsonization* because it uses proteins named *opsonins*), which marks them for destruction by phagocytes. IgM directly activates complement but also has a regulatory role whereby activation of complement enhances specific antibody production.[754]

Finally, IgM is noted not only for its role in immune defense, but also in immune regulation and immunological tolerance (i.e., prevention of an immune response to self-antigens).

Antibody Types, 4: IgD



Not infrequently described as an 'enigmatic' antibody class, *immunoglobulin delta heavy chain* (IgD) accounts for only a small fraction of a percent (0.02%) of antibodies found in blood serum. This may seem surprising given that IgD (with IgM) is the first Ig expressed by B-cells in their developmental history. B-cells release IgD not only in the blood, but through respiratory, salivary, the lacrimal gland above the eyes, and breast milk.[755] The half-life of IgD is relatively short—about 3 days.[756]

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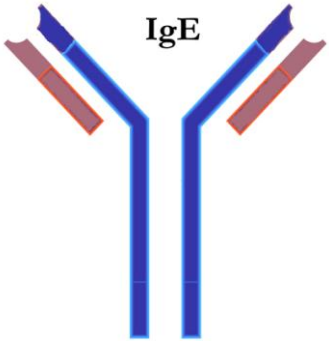
[749] Keyt et al., "Structure, Function, and Therapeutic Use of IgM Antibodies," 6.
[750] Weill and Reynaud, "IgM Memory B Cells," 6.
[751] Blandino and Baumgarth, "Secreted IgM: New Tricks for an Old Molecule," 1023.
[752] Keyt et al., "Structure, Function, and Therapeutic Use of IgM Antibodies," 4.
[753] Keyt et al., "Structure, Function, and Therapeutic Use of IgM Antibodies," 12.
[754] Heyman and Shulman, "Structure, Function, and Production of Immunoglobulin M (IgM)."
[755] Chen et al., "Immunoglobulin D Enhances Immune Surveillance."
[756] Gutzeit, Chen, and Cerutti, "The Enigmatic Function of IgD."

IgD is an Ig that starts as a surface immunoglobulin but can ultimately be expressed as either a membrane-bound Ig (mIgD) or as a secreted Ig (sIgD). As the former, it is found co-expressed with IgM in more than 90% of mature B-cells. As the latter, it is often elevated in people with autoimmune diseases.[757] Alongside IgA and IgG, IgD has 3 constant domains and 1 variable domain. As the illustration shows, the structural appearance of IgD looks like that of IgG. Its specific Fc receptor is IgDR.

IgD promotes immune defense by inducing the activation and infiltration of immune cells, which causes inflammation and subsequent tissue damage as pathogens are destroyed.[758] IgD binds to circulating basophils of the innate immune system. Such basophils, stimulated by the IgD, produce antimicrobial factors. It is thought that B-cells may produce IgD to instruct basophils regarding the antigenic composition of the upper respiratory tract at any given time. In so doing it arms basophils with an adaptive recognition system able to amplify responses to IgD-targeted antigen. [759]

Antibody Types, 5: IgE



Even less than IgD is the trace amount of *immunoglobulin epsilon heavy chain* (IgE)—the least abundant Ig class found in blood serum, where in peripheral blood its half-life is 1–5 days. Interestingly, IgE levels are low at birth, peak at about age 15, and thereafter decline.[760]

In those more prone to allergies (*atopic* individuals) concentrations in circulation can reach over 10 times the levels found in others. Like IgM, IgE has 4 constant domains and 1 variable domain. The extra constant domain (Cε2) is a critical determinant in its distinctive physical structure and unique functions.[761]

In binding to mast cells, IgE is thought to have a role in the allergic reaction commonly called 'hay fever' (*pollinosis*).[762] In fact, IgE is perhaps most well-known for being implicated in allergic reactions. Its one heavy chain variable (HV) domain shows a different set of reactions in allergic individuals—a consequence, perhaps, of allergens acting as 'superantigens' in such people.[763]

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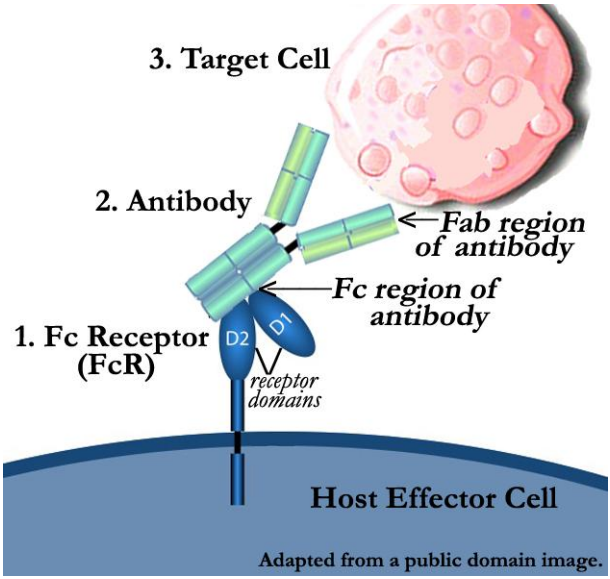
[757] Wu et al., "The Elevated Secreted Immunoglobulin D," 1.
[758] Wu et al., "The Elevated Secreted Immunoglobulin D," 2.
[759] Chen et al., "Immunoglobulin D Enhances Immune Surveillance," and Gutzeit, Chen, and Cerutti, "The Enigmatic Function of IgD."
[760] Mak and Saunders, *The Immune Response*, 119.
[761] Gould et al., "The Biology of IgE and the Basis of Allergic Disease," 581.
[762] Castillo et al., "Seasonal Influence on Serum and Receptor-Bound IgE in Pollinosis." Also see Gould et al., "The Biology of IgE and the Basis of Allergic Disease," 590.
[763] Gould et al., "The Biology of IgE and the Basis of Allergic Disease," 581.

Antibodies interact with other entities (pathogens, immune cells, etc.). Both the *fragment antigen-binding* (Fab) and the *fragment crystallizable* (Fc), which we discussed earlier, have important roles. The illustration below shows how an antibody (2) interacts with an effector cell (1) and a target cell (3).



The Fab region is responsible for ensuring a suitable response occurs to the antigen provoking the host immune system. This region of the antibody binds to the antigen.

As we noted earlier, the Fc is a region that binds to various cell receptors as well as to certain proteins of the *complement system* (or simply *complement*). Host cell receptors—*Fc receptors* (FcRs)—are designed to attach to the Fc region of an antibody bound to either the virus itself or to an infected cell. In this manner an immune response can be activated that stimulates 'devouring cells' (*phagocytes*) and 'poisonous to other cells' (*cytotoxic*) cells to accomplish their functions against pathogens.

Antibody Fragment Antigen-binding (Fab) *Region*

The name tells all: the 'F' in Fab stands for a protein that is a part—'fragment'—of the antibody that recognizes substances (antigens) that present possible danger. The fragment is termed 'antigen-binding'—the 'ab' in Fab—because by it the antibody becomes attached to the pathogen. More specifically, at the tips of the Fab are six hypervariable loops called *complementarity determining regions* (CDRs); a set of CDRs constitutes a *paratope*, the 'place' (*-tope*) 'beside' (*para-*) the corresponding 'place' (*-tope*) 'on' (*epi-*) the antigen—its *epitope*—where binding occurs. Not all attachments are of like nature because the surfaces of antibodies and antigens are irregular, with pockets and grooves as well as flat places, and thus matches are variable. Some have been characterized as like a 'lock-and-key' binding while others are more of a 'handshake' character.[764]

[764] Al Qaraghuli et al., "Antibody-Protein Binding," 1–2. Also Murphy and Weaver, *Janeway's Immunobiology*, 147.

The Fc region offers another functional 'fragment' (F) termed 'crystallizable' (c)— the first domain of immunoglobulin structure to be determined by crystallography. The Fc region of an antibody, like the Fab region, engages in 'binding,' but to a variety of targets to accomplish a range of functions.[765]

Fc recognizes and binds to . . .	**. . . and facilitates**
cell surface receptors of effector cells (FcRs) *and* other cells (Target cells (TCs))	antibody-dependent cellular cytotoxicity (ADCC); antibody-dependent cellular phagocytosis (ADCP)
immune system proteins (Complement)	complement-dependent cytotoxicity (CDC)

In *antibody-dependent cellular cytotoxicity* (ADCC) the Fc domain of an antibody interacts with a particular kind of FcR (FcγR) on an innate immune system effector cell (macrophages, neutrophils, and especially natural killer cells), while it is also binding to viral protein on the surface of an infected cell. This complex interaction pattern induces cytotoxicity of the infected cells.[766]

Like ADCC, in *antibody-dependent cellular phagocytosis* (ADCP) a two-step mechanism operates: the antibody binds to a target cell (TC), and the Fc domain of the antibody binds to FcγR on the surface of an immune cell (or to complement factors). The immune cells are macrophages or other phagocytic cells, which are then activated to destroy the target cell.[767]

Researchers Matthew Zirui Tay, Kevin Wiehe, and Justin Pollara, write that "These Fc receptor-dependent antibody functions provide a direct link between the innate and adaptive immune systems, harnessing the potent anti-pathogen functions of the innate immune system, and overcoming its inherent limited pattern recognition capacity by utilizing the diversity and specificity of the adaptive immune response."[768]

In *complement-dependent cytotoxicity* (CDC) the Fc domain of an antibody activates the complement system. The antibody, already bound to a cell, uses its Fc region to bind to a subcomponent (C1q) of the C1 complement complex. Then recruitment of a series of complement proteins produces a protein complex—the *membrane attack complex* (MAC)—and the resulting *complement cascade* can kill a pathogen, or activate an immune system phagocyte to destroy it.[769]

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[765] Table material relies on various sources, including Choe, Durgannavar, and Chung, "Fc-Binding Ligands of Immunoglobulin G" and those cited in the following paragraphs.
[766] Wang et al. "Quantifying the Contribution of Fc-Mediated Effector Functions," 18002; Van Erp et al., "Fc-Mediated Antibody Effector Functions," 2.
[767] Herbrand, "Antibody-Dependent Phagocytosis."
[768] Tay, Wiehe, and Pollara, "Antibody-Dependent Cellular Phagocytosis," 2.
[769] Wang et al., "Regulation of Antibody-Mediated Complement-Dependent Cytotoxicity."

Cells of the Adaptive Immune System: T-Lymphocytes (T-Cells)

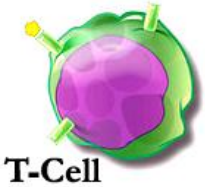

Image courtesy of National Institute of Allergy and Infectious Diseases (NIAID).

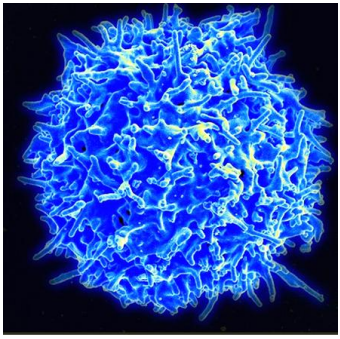

Image courtesy of National Institute of Allergy and Infectious Diseases. (Scanning electron micrograph of a human T lymphocyte.)

As we have seen from looking at B-cells, there are differences between them and T-cells in appearance. Here it is worth pausing to also recall that there are differences between artistic images and photos. The image at the left is an artistic representation of a T-cell; the one at the right is a scanning electron micrograph of an actual T-cell—both are courtesy of NIAID. The value of the artistic drawing is that it makes it easier to recognize structural features. That is why we have been relying on such illustrations throughout this volume. With that reminder, let's start with some basics.

T-Cell Basics: Difference from B-Cells; T-Cell Origin, Maturation, Activation

Unlike B-cells, T-cells do *not* produce antibodies. One way to broadly characterize the difference between T-cells and B-cells is like this:

- *B-cells* are responsible for *humoral (antibody)-mediated immunity*.
- *T-cells* are responsible for *cell-mediated immunity*.

We will explore 'cell-mediated immunity' as we go on.

The 'T' in *T-Lymphocytes*—more familiarly known as *T-cells*—derives from the fact that they (mostly) mature in the *thymus*—a primary lymphoid organ located behind the sternum in the upper front of the chest. T-cells originate from *hematopoietic stem and progenitor cells* (HSPCs) in bone marrow.[770] Then most of them migrate for maturation to the thymus (rather than to the spleen, as typifies B-cell maturation).[771] In the thymus occurs *thymopoiesis* (literally, 'in the thymus' (*thymo-*) 'formed' or 'made' (*-poiesis*)), by which the progenitor cells, now becoming 'thymus-lymphocytes' (*themocytes*), develop into T-cells. This entails the progenitor cells experiencing *T-cell receptor* (TCR) gene rearrangement to generate co-receptor $CD4^+/CD8^+$ double positive (DP) thymocytes. These undergo a selection process into either $CD4^+$ ($CD4^+CD8^-$) or $CD8^+$ ($CD4^-CD8^+$) single positive (SP) thymocytes and enter the periphery.[772]

We can picture this matter of T-cell origin and maturation like this:

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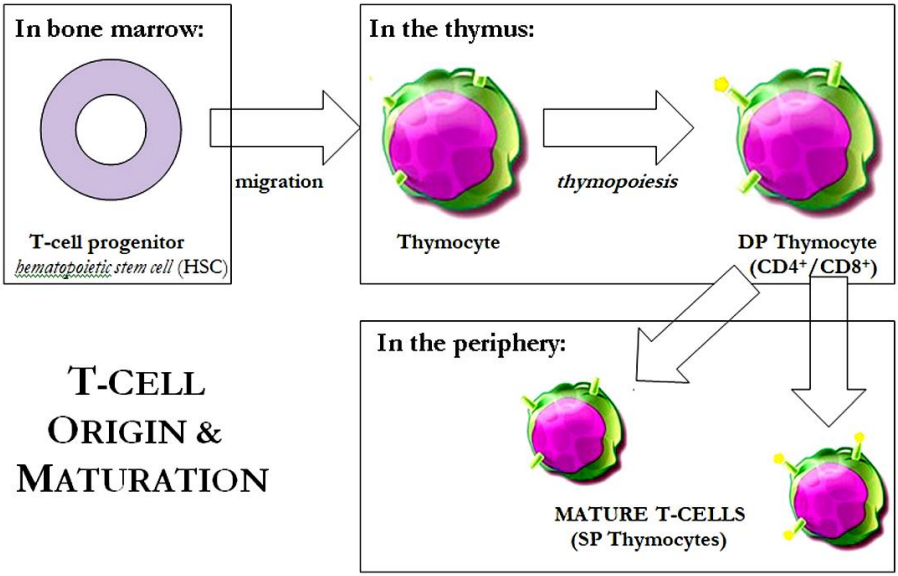
[770] For details, see Yang, Bell, and Bhandoota, "T-Cell Lineage Determination."

[771] Naparstek et al., "Effector T Lymphocyte Lines Migrate to the Thymus and Persist There."

[772] Kumar, "Identification and Characterization of Tissue-Resident Memory T-Cells," 7; also see Kumar, Connors, and Farber, "Human T Cell Development, Localization, and Function." Also see Edgar, Michaels, and Zandastra, "Multi-Objective Optimization," 1.



With this picture in mind let us dive deeper. The precursors to the T-cell are the thymocites—cells structured in the thymus. As noted earlier, T-cell receptor (TCR) gene rearrangement is critical for the development of mature T-cells. TCRs are either αβ or γδ chains (with variable (V), joining (J) and/or diversity (D) gene segments), bonded with five *cluster of differentiation 3* (CD3)—a multimeric protein complex—with subunits (γ, δ, μ, π, and Σ). The CD3 mediates T-cell activation signals. All this leads to a range of possible lineages.[773]

In addition to gene rearrangement, also critical is *thymocyte selection*—which is characterized as either 'positive' or 'negative.' This occurs when thymocytes contact *major histocompatibility complex* (MHC)-*peptide* ligands (thus, pMHC) in the thymus. The pMHC (of two types: pMHC I and pMHC II) occurs on antigen-presenting cells (APCs), including the familiar dendritic cell (DC), as well as *cortical thymic epithelial cells* (cTECs), and *medullary thymic epithelial cells* (mTECs). Those thymocytes that only very weakly interact with pMHC (termed 'low affinity') die, while those that too strongly interact with pMHC ('high affinity') are destroyed—the result of *negative selection*. Like Goldilocks' bowl of porridge must be 'just right,' the affinity of the thymocyte with pMHC must be 'just right' ('intermediate affinity'). As such, *positive selection* can occur, which differentiates $CD4^+/CD8^+$ double positive (DP) thymocytes into either $CD4^+$or $CD8^+$ single positive (SP) thymocytes. [774]

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[773] Luckheeram et al., "$CD4^+$ T Cells: Differentiation and Functions," 2 (§2.1 Lymphopoiesis). For more detail see Cieslak et al., "Blueprint of Human Thymopoiesis."

[774] Luckheeram et al., "$CD4^+$ T Cells: Differentiation and Functions," 2. For more detail on positive and negative selection as induced by interaction with MHC, see Saito and Watanabe, "Positive and Negative Thymocyte Selection"; or for a shorter piece, see Sebzda et al., "Positive and Negative Thymocyte Selection."

After maturation and differentiation, naive T-cells enter the vascular system to circulate, moving into tissues as needed, patrolling for pathogens or attacking them at sites of infection.[775] Their activity after leaving the thymus is dynamic—they recirculate from the blood through *lymph nodes* (LN) into lymphatics and back into the blood. Immunologists Flavian Brown and Shannon Turley highlight the importance of LN in remarking, "the LN serves as a central site for every stage of the T cell life cycle by recruiting naive T cells from the blood, promoting naive T cell survival, providing an environment for T cell differentiation or tolerance, and influencing the homing properties of memory T cells."[776]

In addition to hematopoietic cells like T-cell leukocytes, the lymph nodes contain many other important cells, including those that provide connective tissue (*stromal cells*). One such stromal cell type—follicular dendritic cells (FDCs)—we met earlier in connection with B-cells. Another kind, *fibroblastic reticular cells* (FRCs), concentrated in the so-called 'T-cell zone' of LN, shows several functions that regulate T-cell activity. For example, FRC-derived chemokines and cytokines are instrumental in recruitment of naive T-cells and their survival in LN. They help regulate T-cell differentiation. FRCs also play a role in protecting the system facilitating T-cell movement (e.g., through specialized blood vessels named *high endothelial venules* (HEVs). Additionally, they may play a part in the prevention of autoinflammation by T-cells (a process termed *tolerizing*) and limiting the expansion of T-cell pools.[777]

Now let us join together elements of the preceding paragraphs to focus on *T-cell activation*. Through the process of thymic T-cell development that we have been examining, T-cell receptors (TCRs) come to complete expression, are prepared to avoid becoming stimulated by self-antigens (aided, for example, by FCRs), and join the peripheral pool of T-cells. In this pool, 90% or more of the T-cells express αβTCR (with the remaining 1–10% expressing γδTCR. As indicated earlier, the TCR's αβ chain forms a complex with CD3 subunits (γ, δ, μ, π, and Σ) to become a fully functional TCR on the T-cell's surface. Then the TCR interacts with pHMC—i.e., peptide ligands of the major histocompatibility complex (MHC) on an antigen-presenting cell (APC). Specifically, TCRs recognize antigens as short peptides that have been bound to MHC. After this recognition the T-cell forms close contact with the target cell (TC) in an organized but dynamic organization termed an *immunological synapse* (because it is reminiscent of neuronal synapses in the brain). Although this TCR-pMHC interaction is primary for T-cell activation, it is accompanied by other ligand pairs, some of them of a stimulating nature and others of an inhibitory character.[778]

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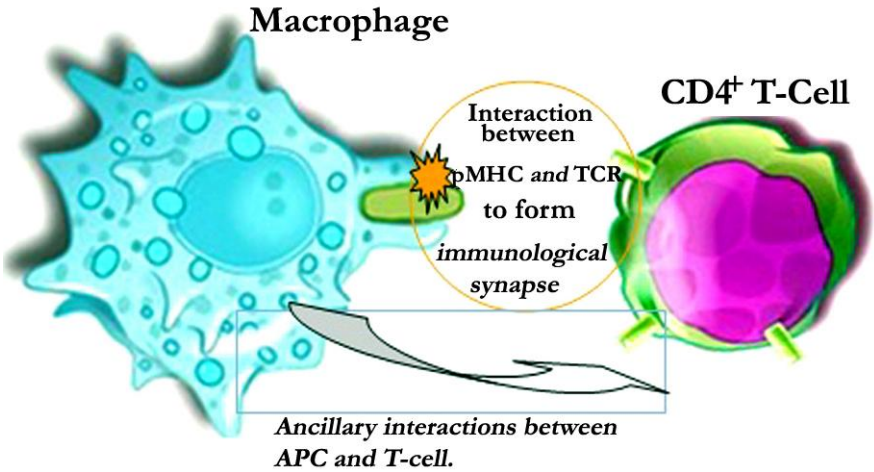
[775] On the larger subject of migrations, see Vuononvirta, Marelli-Berg, and Poobalasingam. "Metabolic Regulation of T Lymphocyte Motility and Migration."
[776] Brown and Turley, "Fibroblastic Reticular Cells," 1389.
[777] Brown and Turley, "Fibroblastic Reticular Cells," 1390–92.
[778] Glatzová and Cebecauer, "Dual Role of CD4 in Peripheral T Lymphocytes," 1–3.

What we've been discussing can be illustrated like this:



Once activated, those T-cells known as 'helper' T-cells then use cytokine messengers to involve B-cells that produce antibodies (as we saw earlier).

The Many Roles of Cytokines

That brings us back to the many roles played by *cytokines* ('cells' (*cyto-*) that 'change' (*-kine*) the behavior or status of other cells). Although we will continue to note their roles as we go along, it seems prudent to pause and again call attention to them in a general manner as we consider the maturation of T-cells and then direct our gaze at T-cell types and their functions.

Cytokines, especially interleukins, occupy key roles from the start. Thus IL-7 supports regulating survival, differentiation, and proliferation of developing *progenitor T-cells* (pro-T).[779] Other interleukins (e.g., IL-1α/β, IL-3, IL-6, IL-12) are found in the thymus, too, though their parts in T-cell development remain unclear. That is true, too, of another cytokine, *tumor necrosis factor* α (TNFα).[780]

When differentiated into helper T-cells, these produce cytokines. In fact, immunologists Jordy Saravla, Nicole Chapman and Hongbo Chi have written that, "Overall, the production of signature cytokines defines T helper cell subsets and functional capacities."[781] Th1's principal cytokines are *interferon gamma* (IFN-γ), *lymphotoxin alpha* (LTα), and *interleukin-2* (IL-2); Th2s produce a number of interleukins (IL-4, IL-5, IL-9, IL-10, IL-13, IL-25); Th17s produce IL-17a, IL-17f, IL-21, and IL-22.[782] We dare not lose sight of cytokines.[783]

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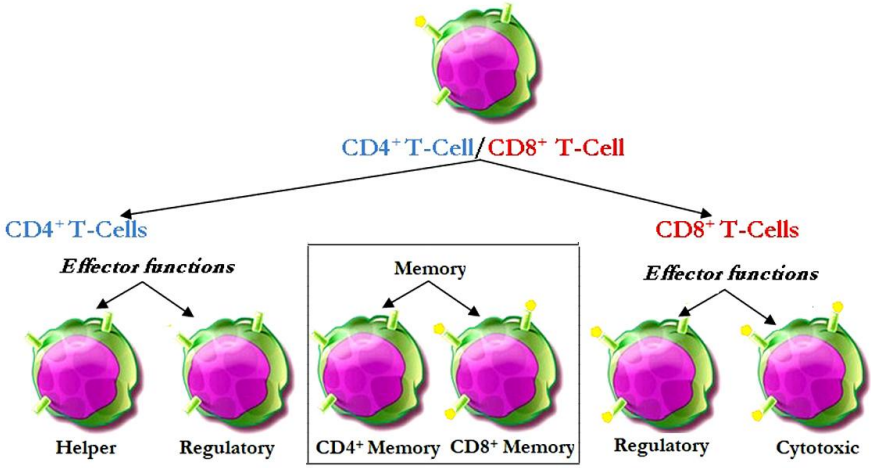
[779] Edgar and Zanstra, "Inflammatory Cytokines Regulate T-Cell Development," 4.
[780] Edgar, Michaels, and Zanstra, "Multi-Objective Optimization," 1.
[781] Saravla, Chapman, and Chi, "Helper T Cell Differentiation," 635.
[782] Zhu and Paul, "CD4 T Cells: Fates, Functions, and Faults," 1559. Also see Miner and Croft, "Generation, Persistence, and Modulation of Th0 Effector Cells."
[783] As one example of a more specialized study, see Cox, Kahan, and Zajac, "Anti-viral CD8 T Cells and the Cytokines They Love."

We have seen the result of the process of maturation culminates in double positive (DP) thymocytes ($CD4^+/CD8^+$) differentiating into single positive (SP) thymocytes ($CD4^+$or $CD8^+$). This matters because it leads to differentiation in function. Mature, but naive, T-cells need antigenic stimulation to acquire functional specificity. $CD4^+$ T-cells achieve this as a result of T-cell receptor (TCR) and CD4 as co-receptor interacting with antigen-MHC II complex, which typically is found on professional APCs (e.g., DCs, B-cells). The consequence of this is that TCR, coupled with CD3 activation, induces signaling pathways that result in naive cell proliferation and subsequently differentiation.[784]

The resulting kinds of T-cells can be very generally pictured as follows:



Differentiation of (most) T-cells often is conveniently distinguished as:

- cytotoxic T-cells ($CD8^+$); and
- helper T-cells ($CD4^+$).

However, the matter of T-cell type is not quite so simple and straightforward as this suggests. Still, this division between T-cells that *attack* (cytotoxic) versus those that *assist* (helper T-cells and others) gets us started.

Focusing on function, T-cells can be divided (alphabetically) into four types:

- cytotoxic T-cells ($CD8^+$);
- helper T-cells ($CD4^+$);
- memory T-cells($CD4^+$ or $CD8^+$); or,
- regulatory (or, suppressor) T-cells ($CD4^+$ or $CD8^+$).

The first two we have already met briefly—and they are the two major types of effector (Teff or T_{EFF}) T-cells. But all four require some further attention.

[784] Luckheeram et al., "$CD4^+$ T Cells: Differentiation and Functions," 2 (§2.2 $CD4^+$ T Cells Activation and Differentiation).

Cells die all the time—it is a part of the reality of human life all through life. The two types of cell death most often discussed and contrasted, because they broadly represent the difference between desired normal processes and less desired, risky ones, are apoptosis and necrosis. Researchers Michele Barry and Chris Bleackley aptly point out, "It is preferable for the host that cells die by apoptosis — which is a 'silent' or non-inflammatory form of death — but necrotic death of individual cells is still better than death of the entire organism."[785] What we need to remember is that many forms of cell death are needed to keep the whole body alive and promote health. Some of these kinds of cell death involve immune system responses and a featured player in this activity are a kind of T-cell.

CD8+ T-cells, also known as *Cytotoxic T-lymphocytes* (CTLs, or T_C), are famously *cytotoxic*—they are 'cells' (*cyto-*) that are 'poisonous' (*-toxic*) to other cells. We shall turn to how they accomplish their mission in a moment. First we must examine such preliminary matters as their differentiation, location, and activation.

For CD8+ T-cells their *terminal differentiation*—their phenotypic changes as they migrate and complete differentiation to become CTLs—requires pro-inflammatory cytokines (e.g., *interleukins* like IL-2 and IL-12).[786]

CTLs in lymphoid organs (e.g., lymph nodes or spleen) are, before priming by antigen-presenting cells (APCs), 'naive.' They simply wander about before contacting an APC (e.g., a dendritic cell (DC)). Recognizing and responding to peptide sequences—specifically *major histocompatibility complex class I* (MHC I) molecules—presented by an APC a CTL is 'activated' and begins the process of differentiation to effector cells. Now, in many infections T-cells, regardless of their specificity, may experience phenotypic changes driven by cytokines—a process termed *bystander activation*. Yet only those T-cells that recognize pathogen-encoded antigen then proceed through multiple rounds of replication. The result are CTL effector progeny—the 'foot soldiers' of the adaptive immune response.[787] It is important to note that while a transient experience of antigen is required for activation, the strength and duration of the antigen, as well as any co-stimulation can affect differentiation and quality of effector function.[788]

Activated CTLs that have recognized antigen on a DC-APC utilize an inflammatory cytokine (CXCR3) to facilitate entry into peripheral tissues.[789] Now armed and ready, they are able to engage in immune defense, including inducing cell death by apoptosis or by secreting substances to accomplish other important ends.

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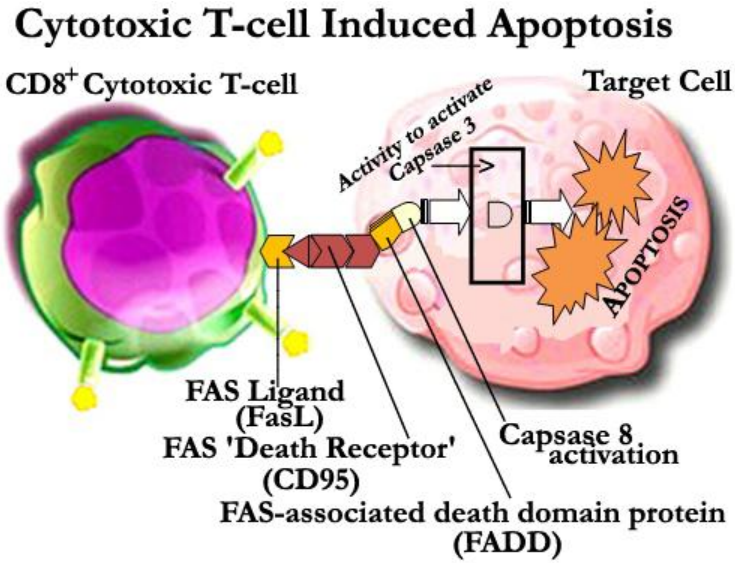
[785] Barry and Bleackley, "Cytotoxic T Lymphocytes: All Roads Lead to Death," 401.
[786] Zhang and Bevan, "CD8+ T Cells: Foot Soliders of the Immune System," 163–65.
[787] Zhang and Bevan, "CD8+ T Cells: Foot Soliders of the Immune System," 161.
[788] Seder and Ahmed, "Similarities and Differences in CD4+ and CD8+," 835.
[789] Zhang and Bevan, "CD8+ T Cells: Foot Soliders of the Immune System," 165.

Clearly CTLs are most famous for their involvement in causing the death of other cells. Interestingly, CTLs are similar to the innate system's natural killer (NK) cells in effector function by relying on granule and Fas cell surface death receptor (FAS)-mediated pathways.[790]

We shall examine the former in a moment. But let's first peek at a *FAS-mediated pathway*. It can be simply depicted like this:



In essence the cytotoxic CD8+ T-cell instructs its target cell (TC) to commit suicide (*apoptosis*). It interacts with TC through a membrane protein that is the *ligand*—binding ingredient—with a transmembrane protein on the TC. The T-cell's protein is called the *FAS Ligand* (FasL) because it binds to the Fas protein (also referred to as CD95, or Apo-1, or TNFRSF6) on the TC. Because the Fas protein receives FasL, it acts as a receptor. It is famously called the 'death receptor' because its activation triggers the pathway leading to cell death.

The Fas protein is transmembrane—it has a portion at the cell surface and a portion below. In the latter (in the cytoplasmic area) is a region of itself called the *death domain* (DD). When FasL interacts with Fas the DD is able to signal and recruit an adaptor protein (one that links together parts to form larger signaling complexes). This adaptor protein, *FAS-associated death domain protein* (FADD) binds and activates *caspase-8* (CASP-8), a protease enzyme that acts to facilitate the process of cell suicide. It can do so in more than one manner; the box in the above illustration is meant to convey a general set of activities either done simply and immediately by CASP-8 or more indirectly, but either way leading to the same end—activation of CASP-3 to induce apoptosis.[791]

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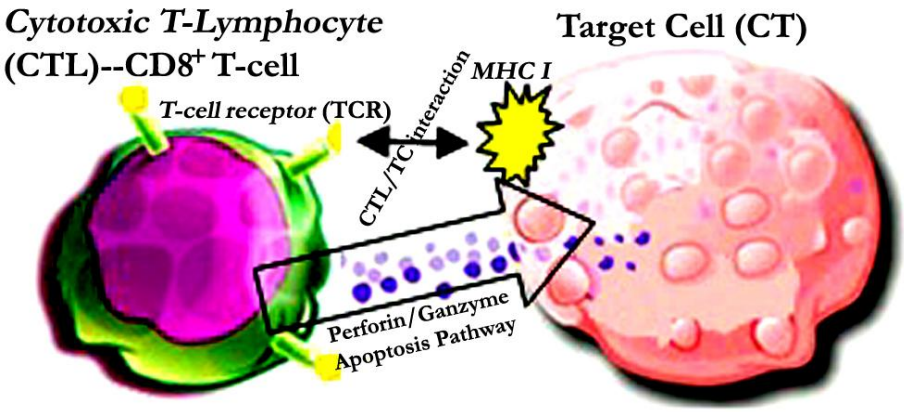
[790] Barry and Bleackley, "Cytotoxic T Lymphocytes: All Roads Lead to Death," 402.

[791] Volpe et al., "Fas-FasL Ligand," 2; also Barry and Bleackley, "Cytotoxic Lymphocytes," 402.

Now we can turn to another way CTL's accomplish their deadly mission. But let's do so in broader context by remembering CTLs effect their work through the production and secretion of various materials. We can quickly summarize these secretions and their effects as follows.

Secretion of . . .		Effect
Kind	*Examples*	
Essential cytolytic mediators	*perforin*; *granzyme*	Perforin/granzyme-induced apoptosis (cell death).
Cytokines	*interferon gamma* (IFN-γ); *interleukin-10* (IL-10) *tumor necrosis factor alpha* (TNF-α)	Enhance antigen presentation and mediate antipathogenic effects (a regulatory role).[792]

The first CTL response—*secreting cytolytic mediators*—intends the cytotoxicity that gives these cells their name and reputation and involves the second of the pathways mentioned earlier. The *perforin/ganzyme apoptosis pathway* is the primary route used by CTLs to eliminate virus-infected cells (or otherwise detrimentally changed cells).[793] This can be simply illustrated, too.



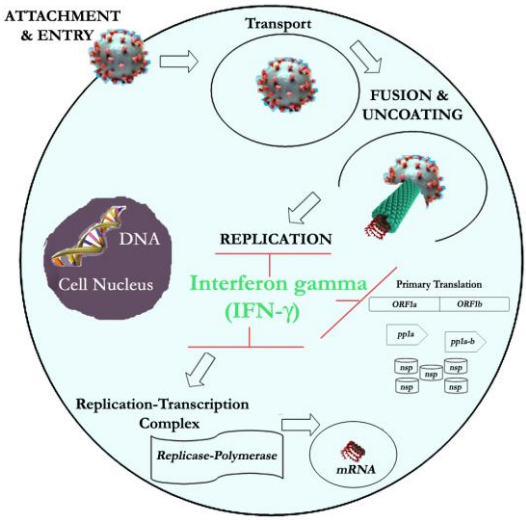
The 'poison' delivered to the enemy cell is contained in very fine particles called *granules* that in interacting with the target cell (TC) are released from inside the CTL (*exocytosis*). The granule contents include the aforementioned perforin and ganzyme. *Perforin* is a protein that binds to the TC membrane, then

[792] Table content derived from Ito and Seishima, "Regulation of the Induction and Function of Cytotoxic T Lymphocytes by Natural Killer T Cell," 1.
[793] For details, see Trapani and Smyth, "Functional Significance of the Perforin/Granzyme Cell Death Pathway."

forms a multimeric complex to cause *tubular lesions*—pore-like channels. *Ganzyme* is an enzyme that cleaves peptide bonds in proteins (thus, a *serine protease*). They enter through the way cleared by perforins and their action induces TC death.[794]

The second matter mentioned in the preceding Table, important but secondary to the matter just covered, is *the secretion of cytokines to enhance antigen presentation and mediate antipathogenic effects*. The first of these—enhancement of antigen presentation—falls to *interferon gamma* (IFN-γ), which accomplishes this task by increasing expression of MHC I, thus making detection of an infected cell more likely. [795]

But IFN-γ also inhibits viral replication. In truth, IFN-γ appears to engage antiviral mechanisms at multiple levels of interaction—entry, replication, gene expression, viral stability, release, and reactivation.[796] Our interest here, though, is in IFN-γ associated with antigen-activated T-cells. Broadly speaking, it can be said to play a crucial part both in establishing an antiviral state and coordinating immune responses related to long-term control of viral infection.[797]



Although it has proven difficult to pinpoint which of the various steps of viral replication IFN-γ targets, several studies suggest the most likely target is viral genome replication. At least with some viruses it has been observed to inhibit both the genomic and sub-genomic RNA syntheses.[798]

The other mentioned goal is the mediation of antipathogenic effects. Toward this end CTLs use *tumor necrosis factor alpha* (TNF-α) and *interleukin-10* (IL-10). The former, TNF-α, can work with IFN-γ to activate innate system macrophages, and through interaction with *tumor necrosis factor receptor 1* (TNFR1) can kill some target cells.[799] The antipathogenic virtues of cytotoxic T-cells has led to their use in immunotherapy.[800]

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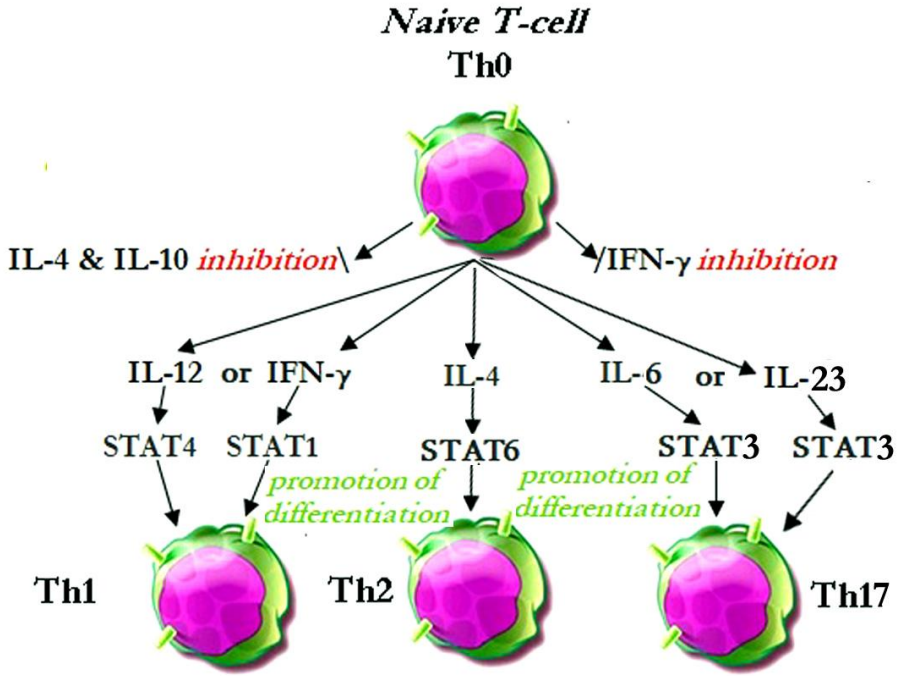
[794] For details, see Peters et al., "Cytotoxic T Lymphocyte Granules."
[795] Actor, *Introductory Immunology*, 59.
[796] Kang, Brown, and Hwang, "Direct Antiviral Mechanisms of Interferon-Gamma," 4 (Fig. 1).
[797] Kang, Brown, and Hwang, "Direct Antiviral Mechanisms of Interferon-Gamma," 2.
[798] Kang, Brown, and Hwang, "Direct Antiviral Mechanisms of Interferon-Gamma," 4.
[799] Murphy and Weaver, *Janeway's Immunobiology*, 392; also see Ito and Seishima, "Regulation of the Induction and Function of Cytotoxic T Lymphocytes by Natural Killer T Cell."
[800] On the subject, see Keller and Bollard, "Immunologic Special Forces."

A *helper T-cell* (Th or T_h) does exactly that; it assists other cells. CD4+ helper cells have been divided into subsets, which include:

- T-helper cell 1 (T_H1 or TH1);
- T-helper cell 2 (T_H2 or TH2);
- T-helper cell 9 (T_H9 or TH9);
- T-helper cell 17 (T_H17 or TH17); and,
- Follicular helper T-cell (T_{FH} or TFH).[801]

The three main subsets are TH1, TH2, and TH17, each specialized for defending against certain kinds of infections.[802]

Differentiation into these subsets is mediated by cytokine involvement.[803] For example, the involvement in differentiating into Th1, Th2, or Th17 involves the following cytokines in either inhibitory or promoting roles as this illustration shows:



Th1 cells are differentiated as such from naive T-cells under the influence of cytokine *interleukin-12* (IL-12) and the cytokine-responsive protein *signal trans-*

[801] See Luckheeram et al., "CD4+ T Cells: Differentiation and Functions."
[802] Gaudino and Kumar, "Cross-Talk between Antigen Presenting Cells and T Cells," 1.
[803] For a general review of the subject, see Saravla, Chapman, and Chi, "Helper T Cell Differentiation."

ducer and activator of transcription 4 (STAT4).[804] Alternatively, the influencing to become TH1 may be by *interferon gamma* (IFN-γ),[805] accompanied by cytokine-responsive protein STAT1. As noted earlier, Th1's principal cytokines are *interferon gamma* (IFN-γ), *lymphotoxin alpha* (LTα), and *interleukin-2* (IL-2).[806] Of these, IFN-γ is principal—and of chief interest to us because of its association with defending against intracellular pathogens like viruses; IFN-γ activates innate immune system cells (macrophages and dendritic cells).[807] TH1 cells mediate immune responses against *intracellular pathogens*, and have been identified as especially important for humans in defense against myobacterial infections (e.g., bacteria that cause tuberculosis or leprosy).[808]

Th2 cells are differentiated as such from naive T-cells under the influence of cytokine IL-4 and cytokine-responsive protein STAT6. Th2s produce a number of interleukins (IL-4, IL-5, IL-9, IL-10, IL-13, IL-25),[809] and like TH1 interact with innate immune system cells by recruiting eosinophils. They also have an impotent role influencing B-cell differentiation and attendant antibody production.[810] Th2 cells are important in the fight against parasitic infections.[811]

Th17 cells are differentiated as such from naive T-cells under the influence of cytokine IL-6 and cytokine-responsive protein STAT3, or IL-23 with STAT3. Th17s produce IL-17a, IL-17f (aka simply as IL-17), IL-21, and IL-22.[812] Although well-known for promoting inflammatory diseases (e.g., multiple sclerosis, psoriasis, and rheumatoid arthritis), Th17s play a role in defense against extracellular bacteria and mucosal fungi.[813]

Follicular helper T-cells (T_{FH} or TFHs) are induced by IL-21 and IL-6, with cytokine-responsive protein STAT3.[814] TFHs operate in secondary lymphoid organs regulating antibody responses to T-cell-dependent antigens in germinal centers (GCs). In other words, they 'help' B-cells do their work by producing IL-21 to regulate B-cell differentiation in the GC and promoting immunoglobulin (Ig) switching related to B-cell affinity maturation.[815]

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[804] Bevington et al., "T Cell Receptor and Cytokine Signaling," 2.
[805] Lionakis and Hohl, "Cell-Mediated Defense Against Infection," 52.
[806] Zhu and Paul, "CD4 T Cells: Fates, Functions, and Faults," 1559. Also see Miner and Croft, "Generation, Persistence, and Modulation of Th0 Effector Cells."
[807] Lionakis and Hohl, "Cell-Mediated Defense Against Infection," 52–53; also Gaudino and Kumar, "Cross-Talk between Antigen Presenting Cells and T Cells," 1.
[808] Zhu and Paul, "CD4 T Cells: Fates, Functions, and Faults," 1559.
[809] Zhu and Paul, "CD4 T Cells: Fates, Functions, and Faults," 1559. Also see Miner and Croft, "Generation, Persistence, and Modulation of Th0 Effector Cells."
[810] Lionakis and Hohl, "Cell-Mediated Defense Against Infection," 53; also Gaudino and Kumar, "Cross-Talk between Antigen Presenting Cells and T Cells," 1.
[811] Gaudino and Kumar, "Cross-Talk between Antigen Presenting Cells and T Cells," 1.
[812] Zhu and Paul, "CD4 T Cells: Fates, Functions, and Faults," 1559, and Lionakis and Hohl, "Cell-Mediated Defense Against Infection," 53 (Fig. 6.2).
[813] Lionakis and Hohl, "Cell-Mediated Defense Against Infection," 55.
[814] Saravla, Chapman, and Chi, "Helper T Cell Differentiation," 635, and Lionakis and Hohl, "Cell-Mediated Defense Against Infection," 53 (Fig. 6.2).
[815] Lionakis and Hohl, "Cell-Mediated Defense Against Infection," 56.

Early in this chapter we noted the importance of *immunological memory*. The adaptive immune system has been distinguished from the innate immune system both by its flexibility of response and by this ability to learn and remember the dangers it encounters. Researchers Sarah Bevington and colleagues capture each aspect and the linkage between them when they observe, "The adaptive immune system relies on both specific antigen (Ag) recognition and on the ability of lymphocytes to maintain a memory of previous encounters with Ags."[816]

An adaptive immune response includes, as we have seen, effector functions such as $CD4^+$ T-cells helping B-cells activate and produce antibodies, or $CD8^+$ cytotoxic cells engaging and destroying pathogens. But as an immune response ends it requires a *contraction phase*—a reduction of response. This contraction is needed to avoid a too large set of long-lived T-cells remaining in circulation. So programmed cell death (*apoptosis*) occurs, which dramatically reduces the number of T-cell survivors.[817] Memory T-cells can be either $CD4^+$ or $CD8^+$ as some of each population of effector cells survive contraction.

After response to a virus some 90% of $CD8^+$ cytotoxic cells will have died in the contraction phase,[818] and various studies indicate the reduction rate of $CD4^+$ effector T-cells is greater and swifter.[819] In essence, effector T-cells prove either destined to a short existence as *short-lived effector cells* (SLECs), or *as memory precursor effector cells* (MPECs) that will become memory T-cells.[820] There are markers that serve as useful predictors of which effector T-cells will perish and which persist. For example, among $CD4^+$ T-cells alterations in the expression of certain cell surface molecules (e.g., CD62L, CD44, CCR7 and CXCR5) serve as markers of a predictable destiny (e.g., those expressing CD44 mark memory T-cells).[821] Among $CD8^+$ T-cells those of $KLRG12^+$ $CD127^{low}$ phenotype typically prove to be SLECs, while those of a $CD127^{hi}$ $KLRG12^-$ phenotype prove more likely destined to become memory T-cells.[822] Of course, as we shall see, the matter is not as easy or straightforward as these remarks may suggest.

When the contraction phase is done the surviving T-cells have entered a quiescent state—they are poised and ready, but not active. Their potent readiness is shown in that when reexposure to their antigen occurs these cells—known as *antigen-specific* (Ag-specific) memory T-cells—are able within the first couple of hours to activate hundreds of immune response genes. [823]

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[816] Bevington et al., "T Cell Receptor and Cytokine Signaling," 1.
[817] On the general topic, see Garrod et al., "Dissecting T Cell Contraction In Vivo."
[818] MacKinstry, Strutt, and Swain, "Regulation of $CD4^+$ T-Cell Contraction" (reporting 2–3 days with influenza virus in view).
[819] Nussbaum and Whitton, "The Contraction Phase," 6611 (with the lymphocytic choriomeningitis virus (LCMV) specifically in view and a time of about 3 weeks (21 days)).
[820] Joshi and Kaech, "Effector CD8 T Cell Development," 1310.
[821] Hojyo et al., "Multiple Developmental Pathways," 589–90.
[822] Renkema et al., "$KLRG1^+$ Memory CD8 T Cells," 1059.
[823] Bevington et al., "T Cell Receptor and Cytokine Signaling," 2.

CD4+ memory T-cells result from effector T-cells surviving contraction, entering a quiescent state until reexposure to antigen, then 'awakening' to rapidly proliferate in fighting the danger. As defined by a number of factors (effector functions, homeostatic migratory patterns, tissue distributions, or capacities for secondary immune responses), CD4+ memory T-cells types are known as:

- *memory T helper cells* (memory Th1, Th2, and Th17);
- *memory follicular helper T- cells* (memory Tfh or memory T_{FH});
- *central memory T-cells* (Tcm) or *effector memory T-cells* (Tem or T_{EM});
- *tissue-resident memory T-cells* (Trm or T_{RM}) or *recirculating memory T-cells* (Trcm or T_{RCM}); and,
- *resting memory CD4 T-cells*.[824]

These memory cells provide an enhanced response because of two important factors: the *antigen-specific memory T-cells are present at higher frequency* than was true for the original naive T-cell population, thus providing for *better surveillance and detection of the pathogen*, and, the *memory T-cells are able to make effector responses more rapidly* than primary (naive) responding cells. The exact nature of the response depends on the type of infection as well as the signals provided by the antigen presenting cell (APC).[825]

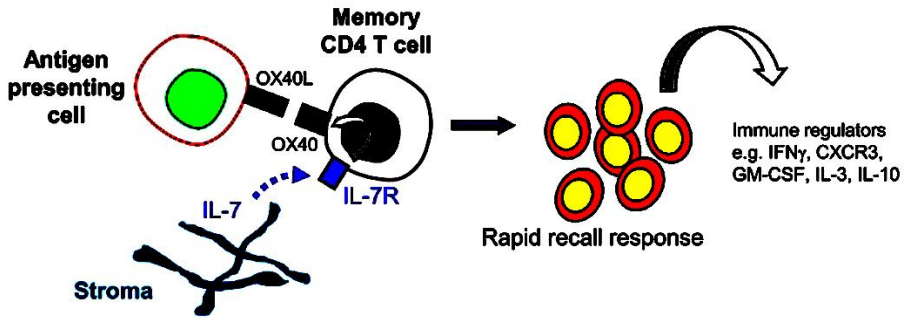
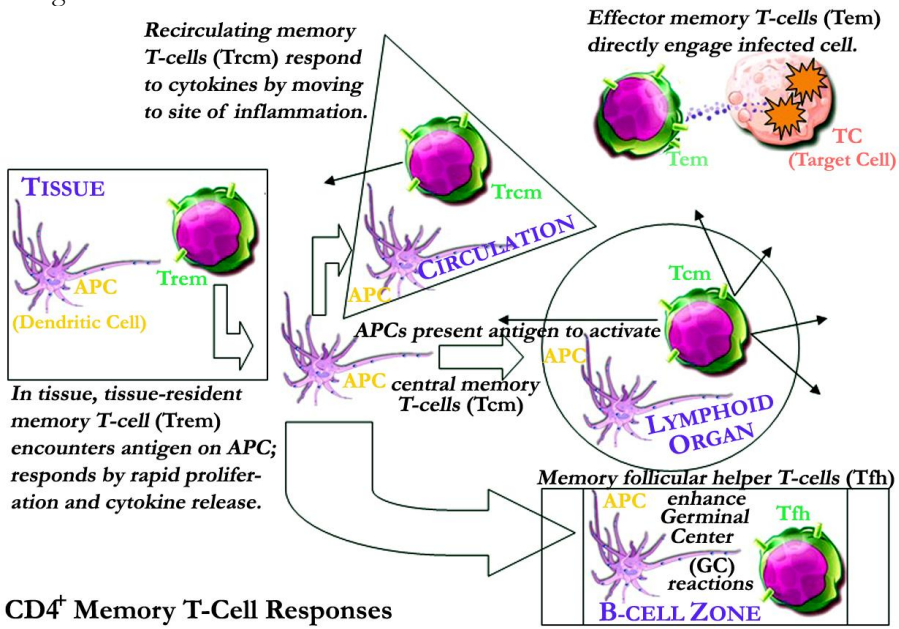


FIGURE 8 | The role of receptor signaling in immunological memory. (A) Long-term immunological memory in CD4 T cells is dependent on cytokine and TNFSF networks involving interleukin 7 (IL-7) and OX40 that maintain the rapid recall response in CD4 memory T cells. Stroma-derived IL-7 upregulates expression of OX40, the receptor for OX40L, which is produced by antigen-presenting cells.

The above illustration is courtesy of Sarah L. Bevington, Pierre Cauchy, David R. Withers, Peter J. L. Lane, and Peter N. Cockerill, "T Cell Receptor and Cytokine Signaling Can Function at Different Stages to Establish and Maintain Transcriptional Memory and Enable T Helper Cell Differentiation." *Frontiers of Immunology* 8 (Mar., 2017) art. 204. doi: 10.3389/fimmu.2017.00204. The material, which appears on page 12, is made available by Creative Commons Attribution License (CC BY), the license for which can be found at https://creativecommons.org/licenses/by/4.0/ legalcode. The original work has been altered as follows: the illustration here is only the excerpt labeled A with its accompanying text.

[824] Hojyo et al., "Multiple Developmental Pathways," 589.

As indicated a moment ago, the response mounted by memory cells is a manifold one. It involves different types of CD4+ memory T-cells, as the following illustration shows.[826]



CD4+ Memory T-Cell Responses

The illustration shows how memory T-cells are placed strategically, and it has become familiar to label the kind of memory T-cell by its location. Thus, T_{REM} memory cells are those found in tissues of the body. Some circulate through the body (T_{RCM}), ready to be called into action where needed. The critically important central memory T-cells (T_{CM}) are in lymphoid organs and when activated are ready to disperse as needed. Memory follicular helper T-cells (T_{FH}) in the B-cell zone are critical, as we saw earlier in the chapter, for success of germinal centers and thereby successful production of antigen-specific antibodies. All these different cells in different places have value.

Because memory T-cells retain characteristics of the activated T-cells from which they are derived they also can be typed based on their functional responses. In this respect, the consequences of rapid cytokine production by them depends on their location, and their diversity in that respect enhances their functional success as they provide protection in distinct ways. Rapid production of the most appropriate cytokine enables them to quickly control a pathogen. Thus, for example, release of IFN-γ protects against viral infections.[827]

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[825] MacLeod et al., "CD4 Memory T Cells: What Are They and What Can They Do?" 2.

[826] This illustration may be compared to those in Gray, Westerhof, and MacLeod, "The Roles of Resident, Central, and Effector Memory CD4 T Cells," 576 (Fig. 1), and MacLeod et al. "CD4 Memory Cells: What Are They and What Can They Do?" 19 (Fig. 1).

[827] Gray, Westerhof, and MacLeod, "The Roles of Resident, Central, and Effector Memory CD4 T Cells," 575.

As noted at the start of this section, there are both $CD4^+$ and $CD8^+$ memory T-cells. Significant differences have been observed between them. For example, $CD4^+$ memory T-cells are not usually abundant after exposure to antigen as is true of $CD8^+$ memory T-cells.[828] Cytokines IL-2 and IL-15 may exert a more profound effect on $CD8^+$ memory T-cells than their $CD4^+$ counterparts leading to a more sustained proliferative capacity for the former.[829] On the other hand, $CD4^+$ memory T-cells appear to preferentially accumulate in mucosal tissues where they outnumber $CD8^+$ memory T-cells. Both $CD4^+$ and $CD8^+$ memory T-cells are found in the skin tissue—a barrier holding a large proportion of the body's tissue-resident memory T-cells (T_{RM})—but they are typically located in different parts, with $CD8^+$ T_{RM} in the epithelium and $CD4^+$ T_{RM} in the underlying dermis tissue.[830] Finally, it is worth noting that $CD4^+$ memory T-cells assist in important ways the development, optimization, and maintenance of $CD8^+$ memory T-cells.[831]

$CD8^+$ effector cells that survive the contraction phase's cell-dying (*apoptosis*) can develop into *resting memory cells*.[832] $CD8^+$ memory T-cells are not all of one nature. It has been commonplace to distinguish them by their *location* as:

- *central memory T-cells* (Tcm or T_{CM});
- *effector memory T cells* (Tem or T_{EM});
- *peripheral memory T-cells* (Tpm or T_{PM}); and,
- *tissue-resident memory T-cells* (Trem or T_{RM})

Central memory T-cells (T_{CM}) are localized to secondary lymphoid tissue but can be found within circulation, too.[833] Circulating T_{CM} cells function as first responders against infections in peripheral tissues.[834] Effector memory T cells (T_{EM}) circulate through non-lymphoid tissues, show a decreased ability compared to T_{CM} cells to traffic to lymphoid tissues, and often display a lower capacity to proliferate to antigen (Ag).[835] While both T_{CM} and T_{EM} can be found in the spleen, the former is principally located in the lymphoid tissue that accounts for about a quarter of the spleen and called 'white pulp,' while T_{EM} is found in the 'red pulp' engorged with blood.[836] Peripheral memory T-cells (T_{PM}) have been the most recently identified subtype (with three subsets), and show characteristics that somewhat overlap with both T_{EM} ($CX_3CR_1^{hi}$ subset) and T_{CM} cells ($CX_3CR_1^-$ subset).[837] In absence of antigen encounter they give rise to T_{CM} cells,

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828 MacLeod et al. "CD4 Memory Cells: What Are They and What Can They Do?" 3.
829 Seder and Ahmed, "Similarities and Differences in $CD4^+$ and $CD8^+$," 837.
830 Schreiner and King, "$CD4^+$ Memory T Cells," 2–3.
831 See, for example, Ahrends et al., "$CD4^+$ T Cell Help."
832 Seder and Ahmed, "Similarities and Differences in $CD4^+$ and $CD8^+$," 839–40.
833 Samji and Khanna, "Understanding Memory $CD8^+$ T Cells," 6.
834 Osborn et al., "Central Memory $CD8^+$ T Cells," 14.
835 Joshi and Kaech, "Effector CD8 T Cell Development," 1310.
836 Samji and Khanna, "Understanding Memory $CD8^+$ T Cells," 6.
837 Martin and Badovinac, "Sifting through $CD8^+$ T Cell Memory," 1184. Also see Böttcher et al., "Functional Classification of Memory $CD8^+$ T Cells by CX_3CR_1 Expression."

while with such an encounter they differentiate into T_{EM} cells.[838] Tissue-resident memory T-cells (T_{RM}) remain in the tissues where the primary reaction occurred;[839] they neither reenter circulation nor are found in lymphoid organs.[840]

However, despite what has just been presented, and although there are indications of which effector cells are more likely to eventually become memory cells, the whole matter of $CD8^+$ cell differentiation and resulting subsets is rather more complex than noting cell surface markers and location. It has been noted that the classification of $CD8^+$ memory T-cells into the four subsets listed, while useful for understanding the overall picture of immune surveillance and distinctions of function still does not capture completely the observed diversity within this memory cell pool. Additional subsets have been suggested (e.g., based on expression of CD27 and CD43).[841]

Accordingly, it has been suggested we think in terms of a continuum with those T-cells harboring greater memory cell potential at one end and terminally differentiated effector cells at the other. Between these poles exist many effector T-cells in intermediate differentiation states.[842] For example, as noted earlier, $CD8^+$ T-cells of the $KLRG12^+$ $CD127^{low}$ phenotype are usually characterized as short-lived (SLECs) expected to experience apoptosis in the contraction phase. Although the vast majority of such effector T-cells do die during the contraction phase, a fraction remain in circulation as *long-lived effector cells* (LLECs). Such T-cells demonstrate properties of both SLECs and memory cells, balancing the role of the former's rapid functionality with the latter's longevity. Such observations challenge an easy distinction between SLEC and memory cell.[843]

Compared to the same antigen-specific, *non-memory* T-cells, $CD8^+$ memory T-cells demonstrate all of the following advantages:

- persist in greater numbers;
- populate peripheral organs;
- immediately proliferate, execute cytotoxic functions, and secrete effector cytokines upon antigen (Ag) re-encounter;
- persist long-term (effector cells undergo robust contraction); and,
- exist in different metabolic, transcriptional, and epigenetic states.[844]

Newly created $CD8^+$ memory T-cells are antigen-*independent* but cytokine-*dependent*. The cytokine IL-7 promotes cell survival while IL-15 regulates cell proliferation and self-renewal.[845] Cytokine experience varies among subtypes.

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[838] Allegre, "The Tpm Subset of the $CD8^+$ T Cell Memory Family," 849.
[839] For a comprehensive overview see Mami-Chouaib and Tartour, *Tissue Resident Memory T Cells*.
[840] Samji and Khanna, "Understanding Memory $CD8^+$ T Cells," 6.
[841] Martin and Badovinac, "Defining Memory CD8 T Cell," 4.
[842] Cui and Kaech, "Generation of Effector $CD8^+$ T Cells," 2. Renkema et al., "$KLRG1^+$ Memory CD8 T Cells," 1060, caution, "the increasingly evident heterogeneous nature of memory T cells suggests that establishing distinct cell subsets with rigid definitions may be misguided."
[843] Renkema et al., "$KLRG1^+$ Memory CD8 T Cells," 1060–66.
[844] Martin and Badovinac, "Defining Memory CD8 T Cell," 1.
[845] Kaech and Cui, "Transcriptional Control of Effector and Memory $CD8^+$ T Cell," 2.

The following illustrates a taste of this variation.[846]

Central Memory (T_{CM})	Effector Memory (T_{EM})	Peripheral Memory (T_{PM}) (CX_3CR_1)	Tissue-Resident Memory (T_{RM})
Activated $CD8^+$ T-cells exposed to IL-15 acquire a T_{CM} phenotype; after activation they initially express IL-2 but stop doing so once differentiated into effectors. Maintenance depends on ILs-2, 7 or 15.	Express *chemotactic cytokine* ('chemokine') *receptors*; produce cytokines associated with effector function (IFN-γ and IL-4).	Three T_{FM} subsets with differing functionality and production of IL-2. The $CX_3CR_1^-$ subset contains the most polyfunctional cells, producing IL-2, IFN-γ, and TNF-α.	Responsiveness to TGF-β is typically needed for T_{RM} development (also IL-15, IL-33, and TNF for formation of T_{RM} in skin); maintenance of T_{RM} cells appears to depend on IRF4; T_{RM} produce IFN-γ.

Variation appears to be the order of the day in many respects. The illustration below provides a sense of how memory T-cells differ from their naive and effector predecessors, and how they experience changes across time.

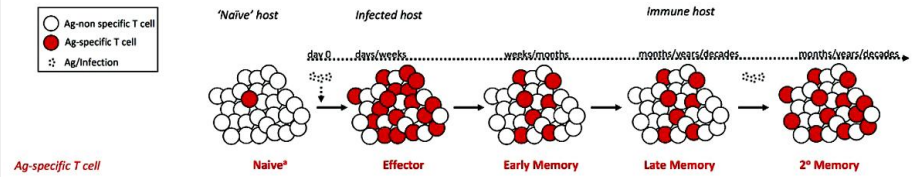


FIGURE 1 | Phenotypic and functional changes within the circulating Ag-specific CD8 T cell pool with time after infection and with additional Ag encounters. Following infection or vaccination, rare naïve CD8 T cells that recognize their cognate Ag robustly proliferate and give rise to an effector CD8 T cell population. Following contraction of the effector pool, memory CD8 T cells are stably maintained at numbers greater than the naïve pool. Upon re-infection or booster vaccination, primary memory CD8 T cells proliferate and generate a secondary effector and memory CD8 T cell pool that is larger in size than the primary memory pool.

Illustration from Matthew D. Martin and Vladimir P. Badovinac, "Defining Memory CD8 T Cell." *Frontiers in Immunology* 9 (Nov., 2018) art. 2692. doi: 10.3389/fimmu.2018.0269. Material is made available by Creative Commons (CC BY) Attribution license (https://creativecommons.org/licenses/by/4.0/ legalcode). The illustration here crops Fig, 1 on page 5, retaining only the top text and images, plus the first lines of the Figure title and explanation.

[846] With respect to Table information, in general see Cox, Kahan, and Zajac, "Anti-viral CD8 T Cells and the Cytokines They Love", and see Martin and Badovinac, "Defining Memory CD8 T Cell." For T_{CM} see Samji and Khanna, "Understanding Memory $CD8^+$ T Cells," 6–7. For T_{EM} also see MacLeod et al. "CD4 Memory Cells: What Are They and What Can They Do?" 2. For T_{PM} see Gerlach et al., "The Chemokine Receptor CX3CR1," 1271.

Context is everything in properly understanding a matter, and with respect to so-called 'regulatory' T-cells the context returns us to another basic immunological idea. The human immune system must distinguish between what properly belongs to the body—'self'—and what does not—'non-self.' There are materials both of the self and of the non-self that can provoke immune response and are thus properly termed 'antigens.' The body's lymphocyte defenders must learn not to respond to self-antigens lest these body cells die by 'friendly fire.' The concept of *self-immune tolerance* requires these defenders not only refrain from acting against self-antigens ('tolerance'), but remove cells that refuse to learn this lesson and act as offenders (*autoreactive cells*).

This task proves easier told than accomplished; some autoreactive lymphocytes escape vigilant detection and make their way to the periphery where they can work harm. *Regulatory T-cells* (T_{reg})—formerly referred to as *suppressor T-cells*—are vital in controlling such undesirable occurrences. Regulatory T-cells play key roles in control of immune responses, limiting chronic inflammatory diseases (e.g., asthma, inflammatory bowel disease (IBD)), and preventing autoimmune diseases (e.g., Type 1 diabetes), while maintaining peripheral immunological tolerance.[847]

Four *basic modes of suppressive action* have been identified for T_{reg} cells.

Inhibitory Cytokines	**Cytolysis**	**Metabolic Disruption of Effector (T_{EFF}) Cells**	**Modulation of Dendritic Cell (DC) Maturation or Function**
IL-10, IL-35, and TGF-β act as suppressive mediators.	Secretion of granzyme A and perforin, or granzyme B-mediated, to induce apoptosis in target cells (e.g., T_{EFF} cells).	T_{reg} cells may, by high 'consumption' of IL-2 thereby 'starve' T_{EFF} cells of the IL-2 they need to survive. Also, involvement in processes suppressing T_{EFF} cell by activating A2A receptor.	T_{reg} cells may interact with DCs in such a way as to interfere with the normal manner by which DCs activate T_{EFF} cells.[848]

In the remarks that follow these modes are given some elaboration.

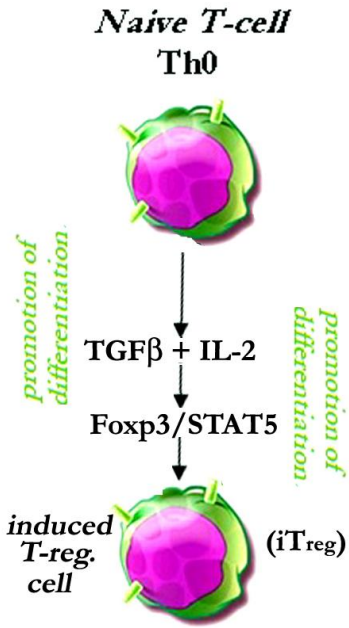
[847] Vignali, Collison, and Workman, "How Regulatory T Cells Work." Also see Mishra et al., "CD8+ Regulatory T Cell—A Mystery to Be Revealed," 1.

[848] Following the order and description in Vignali, Collison, and Workman, "How Regulatory T Cells Work," 2–6.

T_{reg} cells may be of either $CD4^+$ or $CD8^+$ type. We shall begin with $CD4^+$ regulatory cells. A gene named *forkhead box P3* (FOXP3), which has been called 'the peacekeeper of the immune system,' is instrumental in $CD4^+CD25^+$ T-cell regulatory (T_{reg}) function, and FOXPs's high and stable expression in effector (T_{EFF}) T-cells leads to acquisition of T_{reg} phenotype and functions.[849] The gene's protein product, *scurfin* (also referred to as FOXP3), is a forkhead-family transcription factor that regulates T-cell function through inhibition ('negative regulation')—hence the early sense of being a 'suppressor.'[850]

$CD4^+$ T_{reg} cells can be categorized into two groups by point of origin:

- *thymus-derived T_{reg}* (tT_{reg}) cells, and
- *peripherally derived T_{reg}* (pT_{reg}) cells.[851]



The former are also known as *natural regulatory T-cells* (nT_{reg}), because they emerge from the thymus already differentiated as such. The latter are subject to differentiation and popularly known as *induced regulatory T-cells* (iT_{reg}).[852] These are induced from naive Th0 cells by the cytokine *transforming growth factor beta* (TGF-β) and cytokine-responsive protein STAT5 (see at left).[853]

Continuing study has found that certain differences among $CD4^+$ T_{reg} cells exist and suggest further ways to classify them. Thus, while $CD4^+$ $CD25^{high}CD127^{low}$ cells, by virtue of high expression of FOXP3, have been called 'bona fide' T_{reg} cells, others expressing immunosuppressive activity include:

- *effector T_{reg}* (eT_{reg}) cells, and
- *resting T_{reg}* (rT_{reg}) cells.[854]

The suppressive functions of T_{reg} cells may be facilitated by their interactions with effector T-cells (T_{EFF}) or dendritic cells (DCs). T_{reg} cells may inhibit the maturation of DCs, which in turn leads to interference activating T_{EFF} cells, or suppress T_{EFF} cells by high consumption of the IL-2 they need. But there are other ways T_{reg} cells can create effects, such as

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[849] Passerini et al., "*Forkhead Box P3*," 130.

[850] For more on the matter, see Fontenot, Gavin, and Rudensky, "Foxp3 Programs the Development and Function of $CD4^+CD25^+$ Regulatory T Cells."

[851] Li et al., "FOXP3 Regulatory T Cells," 558.

[852] Zhu and Paul, "CD4 T Cells: Fates, Functions, and Faults," 1557.

[853] Lionakis and Hohl, "Cell-Mediated Defense Against Infection," 53 (Fig. 6.2).

[854] Li et al., "FOXP3 Regulatory T Cells," 558. The eT_{reg} are $CD4^+$ $CD45RA^-$ $FOXP3^{high}$ cells, while the rT_{reg} are $CD45RA^+$ $FOXP3^{low}$ cells.

releasing immunosuppressive cytokines (IL-10), disrupting target cell metabolism, or inducing apoptosis.[855]

In an earlier Table about $CD8^+$ we saw that in addition to its famous effector role as a killer of pathogens it also has a regulatory effector function. Simply, its 'effect' is a contribution to the regulation of the immune response in order to protect the body from over-reaction that would create excessive tissue damage. $CD8^+$ T_{reg} cells were described for the first time more than a half century ago (1970), and were initially called 'suppressor T lymphocytes' because of their inhibitory activity. In general they are viewed as a highly heterogeneous class of T-cells which express a variety of surface markers depending upon the environment in which they are found and their inhibitory function therein.[856] $CD8^+$ T_{reg} cells have proved an extraordinarily diverse lot (e.g., $CD8^+CD25^+Foxp3^+$, $CD25^+CD122^+Foxp3^+$ and $CD8^+CD28^-$).[857] In fact, as we found for $CD4^+$ regulatory cells, $CD8^+$ T_{reg} cells show heterogeneity in plasticity of lineage, subtypes, and mechanisms of suppression.[858]

Like $CD4^+$ T_{reg} cells, $CD8^+$ regulatory T-cells exhibit different modes of action. For example, they secrete the immunosuppressive cytokine *interleukin-10* (IL-10) to prevent excessive tissue damage.[859] It has been posited that perhaps different subsets of $CD8^+$ T_{reg} cells are context-dependent in their immune reaction and utilize different inhibitory mechanisms. Thus, some secrete IL-10 to exert inhibitory activity while others may use direct perforin-mediated cytolytic activity against targeted cells.[860] Principal targets of $CD8^+$ T_{reg} cells for destruction are antigen-activated $CD4^+$ T-cells and T_{FH} cells.[861] We can capture some sense of this diversity in the following Table (based on murine studies).

$CD8^+$ T_{reg} Cell Subset	**Function**
$CD8^+Foxp3^+$	suppress T_{EFF} responses and immune rejection
$CD8^+CD103^+$ (vitro)	suppress immune rejection
$CD8^+CD103^+$ (tumor)	negative correlation with tumor control
$CD8^+CD28^+$	suppress T_{EFF} responses
$CD8^+CD122^+$ $CD49d^+$	suppress immune rejection
$CD8^+CD122^{hi}$ $Ly49^+$	suppress Germinal Center (GC) reaction[862]

In sum, T_{reg} cells 'regulate' by exercising supervisory control through inhibiting or suppressing immune reactions as needed to maintain overall health.

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855 Li et al., "FOXP3 Regulatory T Cells," 559–60.
856 Vieyra-Lobato et al., "Description of $CD8^+$ Regulatory T Lymphocytes," 1.
857 Zhang, Wu, and Wang, "Immune Regulation by $CD8^+$ Treg Cells," 805.
858 Mittrücker, Visekruna, and Huber, "Heterogeneity in the Differentiation and Function of $CD8^+$ T Cells," 454–55.
859 Zhang and Bevan, "$CD8^+$ T Cells: Foot Soldiers of the Immune System," 165.
860 Lu and Cantor, "Generation and Regulation of $CD8^+$ Regulatory T Cells," 404.
861 Mishra et al., "$CD8^+$ Regulatory T Cell—A Mystery to Be Revealed," 3.
862 Mishra et al., "$CD8^+$ Regulatory T Cell—A Mystery to Be Revealed," 2 (Table 1).

Whereas B-cells are noted for doing one particular thing—producing antibodies—and doing it very well, their diversity lies within that specialized productivity. On the other hand, T-cells exhibit diversity in a variety of ways, beginning with their division into $CD4^+$ T-cells and $CD8^+$ T-cells, which only name the most prominent types when considering the expression of certain surface markers.

Their diverse nature extends to their life-cycle. Let us consider here only the fates of those who become mature, but naive T-cells. Below are two courses, with the vast majority experiencing the first one.

- Naive T-cells are activated by antigen presentation, undergo clonal expansion to acquire effector functions, and die afterward.
- Naive T-cells are activated by antigen presentation, undergo clonal expansion to acquire effector functions, but survive the contraction phase to become memory T-cells with their own maturation

(It has been noted some memory T-cells form even in the absence of contraction so the link between these two events should not be overemphasized.[863])

We emphasized diversity in function. That of *cytotoxic T-cells* ($CD8^+$) is to kill pathogens. The function of *helper T-cells* ($CD4^+$), as we have seen in examining B-cells, is to facilitate the functions of other immune system cells (e.g., B-cells, macrophages, and cytotoxic T-cells). *Memory T-cells* (both $CD4^+$ and $CD8^+$) are like memory B-cells—they are long-lived repositories of learned response to antigen and when that antigen is reintroduced these cells initiate a process resulting in sizable numbers of effector T-cells. The first two types of T-cells—cytotoxic and helper—as well as the last, regulatory, are all effector T-cells (i.e., they respond to the effect of a pathogen by creating effects of their own). *Regulatory T-cells* (both $CD4^+$ and $CD8^+$) do this in a kind of mopping-up operation to stop autoimmune response, thus limiting damage after the danger has passed.

$CD4^+$ T-cells and $CD8^+$ T-cells obviously share many similarities. But as we have glimpsed, they also possess distinctive differences. We noted earlier, for example, the differences that occur in the contraction phrase related to reducing the number of T-cells and leaving long-lived effector T-cells and memory T-cells. But differences exist even from much before that part of the T-cell life-cycle. $CD8^+$ T-cells develop more readily into effector cells after short-term primary stimulation, seem to require less antigen exposure to launch proliferation, divide sooner, and show a faster rate of cell division. Murine studies have found the frequency of virus-specific $CD8^+$ IFN-γ-producing cells is substantially higher than that for $CD4^+$ IFN-γ-producing cells, both at the peak and throughout the memory response.[864]

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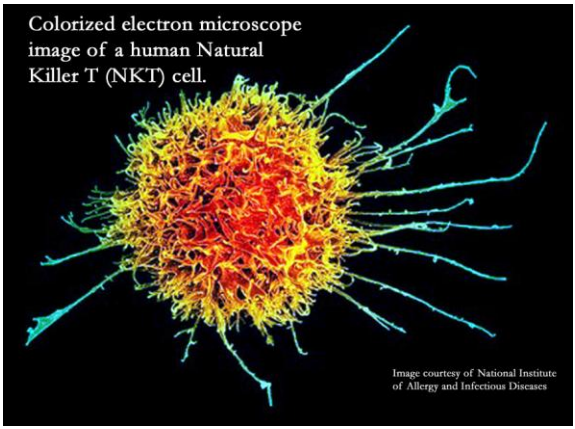
[863] On both the life-cycle points and the caution about linking contraction and memory T-cell formation, see Samji and Khanna, "Understanding Memory $CD8^+$ T Cells," 2.

[864] Seder and Ahmed, "Similarities and Differences in $CD4^+$ and $CD8^+$," 835–36.

Cells of the Adaptive Immune System: Natural Killer T-Cells

Finally, we come to our last major player in the immune system defense forces, one unique in the manner that it bridges the innate and adaptive immune systems: *natural killer T-cells* (NKTs). The 'T' in the abbreviation keys to the 'T-cell' identity in the name—and helps explain why they are placed here in our examination of the immune system. NKTs are not infrequently confused with natural killer (NK) cells of the innate immune system. Though they share some properties of NK cells as well as of other T-cells, NKTs are a distinct type and merit separate attention.



NKTs' uniqueness among lymphocytes lies in structurally bridging innate and adaptive immune systems by expressing both T-cell receptors (TCRs) and NK receptors.[865] We'll return to this point shortly.

NKTs are divided into two basic types. Here are starting notes for each.[866]

	Type I (Invariant)	Type II
Receptors	Vα24Jα18 TCR α-chain NKR-P1C (NK receptor)	Relatively diverse TCR α-& β-chains NKR-P1C (NK receptor)
Recognize	αGalCer lipids presented by CD1d molecules (self- and foreign lipid antigens)	different lipids presented by CD1d molecules (self- and foreign lipid antigens), but not αGalCer lipids
Activated by . . .	[1] antigen presentation [2] CD1d presentation of endogenous ligand(s) [3] APC presentation of inflammatory cytokines (IL-12, IL-18) when stimulated by TLR agonists [4] combination of #s 2 & 3 above	TCR stimulation by antigen presentation (acidification—pH5 or 6—enhances activation); independent of cytokine IL-12
Function	Diverse effector functions against microbial pathogens (e.g., viruses, bacteria, fungi, and parasites)	Like innate arm of adaptive immune system—inhibit proinflammatory functions of other immune cells

[865] Ito and Seishima, "Regulation of the Induction and Function of Cytotoxic T Lymphocytes by Natural Killer T Cell," 1.

[866] Table information derived from variou sources, including Tupin, Kinjo, and Kronenberg, "The Unique Role of Natural Killer T Cells;" Brennan, Brigl, and Brenner, "Invariant Natural Killer T Cells," and Dasgupta and Kumar, "Type II NKT Cells."

Let's break this Table down a bit. NKTs are distinctive in expressing receptors encoded by a *Vα24 gene segment* (Vα24*i*)—a gene evolved from a common ancestor ('orthologous'). They are either $CD4^+$ or $CD4^-$ $CD8^-$, with a range of TCR variable region genes, and as noted above, also a NK receptor (NKR-P1C). Unlike other T-cells, NKTs are *CD1-restricted*, meaning they only respond to lipid (not peptide) antigens on *cluster of differentiation 1* (CD1) family of glycoproteins presented by an antigen-presenting cell (APC).[867]

As we might remember from earlier, T-cell receptors (TCRs) are either αβ or γδ chains (with variable (V), joining (J) and/or diversity (D) gene segments). Professor of Pathology and Laboratory Medicine Jeffrey Actor remarks, "It is now generally accepted that the term 'NKT cells' refers primarily to CD1-restricted T cells coexpressing a heavily biased, semi-invariant TCRα chain paired with one of three TCRβ chains."[868] Type I and Type II NKTs differ significantly in the range of their α- and β-chain pairings. Type I NKTs—*invariant NKTs* (iNKTs)—mostly use Va24-Ja18 TCR α-chain paired with Vβ11. Like other T-cells, they appear to experience some modulation in TCR repertoire after arrival at peripheral organs, with an increased proliferation of lymph node iNKTs associated with a distinct TCRβ repertoire.[869] Type II NKTs express more diverse α- and β-chains, and show features of both conventional and innate-like T-cell.[870]

Type I and Type II NKTs also differ in the antigens they recognize. Type I—iNKTs—recognize *alpha-galactosylceramide* (aGalCer), a specific ligand of the lipid-binding MHC class I-like protein CD1d, and sometimes called 'the model glycosphingolipid antigen.' [871] Type II NKTs, in contrast, are not reactive to αGalCer, but recognize different lipid antigens of two major subclasses (*sphingolipids* and *glycerolipids/phospholipids*).[872]

Type I NKTs are usually divided into three subsets (two others have been proposed), though discussed in different ways in various contexts. They are named based on *lineage* (NKT1, NKT2, NKT17)—analogous to helper T-cell sets. This also links the subsets to expression of *signature transcription factors*—those proteins associated with each lineage (NKT1 (T-bet$^+$); NKT2 ($PLZF^{hi}$); NKT17 (RORγt$^+$).[873] To this can be added association with *cluster of differentiation antigens*—the cell-surface molecules expressed ($CD4^+$, $CD8^+$, or $CD4^-$ $CD8^-$ (or, DN)). These roughly match up to subsets (DN cells having a T_H1 bias, and some $CD4^+$cells match iNKT1; $CD4^+$cells having a T_H2 bias match iNKT2).[874]

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[867] Niemeyer et al., "Natural Killer T Cell Characterization through Gene Expression," 45.
[868] Actor, *Introductory Immunology*, 61.
[869] Jimeno et al., "Tissue-Specific Shaping of the TCR Repertoire."
[870] Cameron et al., "Antigen Specificity of Type I NKT Cells," 4604.
[871] Niemeyer et al., "Natural Killer T Cell Characterization through Gene Expression," 45.
[872] Dasgupta and Kumar, "Type II NKT Cells," 665–67.
[873] Kwon and Lee, "Lineage Differentiation Program" (esp. p. 371); also Jimeno et al., "Tissue-Specific Shaping of the TCR Repertoire," and Hogquist and Georgiev, "Recent Advances."
[874] Krovi and Gapin, "Invariant Natural Killer T Cell Subsets," 11.

Let's further detail these types, but with attention to cytokine profiles.

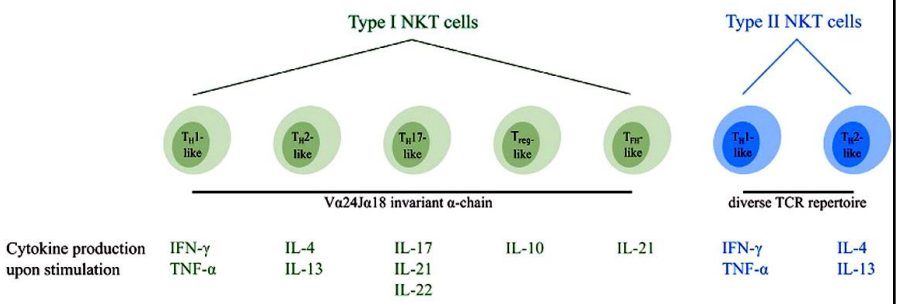


Figure 1 | Overview of the different functional human NKT cell subsets. CD1d restricted human NKT cells can be divided into subsets based on their TCR repertoire and cytokine profile. Type I NKT cells express the invariant Vα24Jα18 TCR α-chain and can be subdivided into five distinct functional subsets (indicated in green). In addition, type II NKT cells express a diverse TCR repertoire and can be subdivided into two functional subsets (indicated in blue). Upon activation, NKT cells secrete a unique pattern of cytokines, indicated for each subtype. Type I and type II NKT cells are able to switch between different functional subsets upon interactions within the TME. Abbreviations: NKT, natural killer T; TCR, T cell receptor; TH, helper T; Treg, regulatory T; TFH, follicular helper T; TME, tumor microenvironment.

(The illustration is from Daniëlle Krijgsman, Marianne Hokland, and Peter J. K. Kuppen. "The Role of Natural Killer T Cells in Cancer—A Phenotypical and Functional Approach." *Frontiers in Immunology* 9 (Feb., 2018) art. 367. doi: 10.3389/fimmu.2018.00367. Material is from page 3 and made available by Creative Commons Attribution (CC BY) License (https://creative commons.org/licenses/by/4.0/ legalcode). No changes have been made except for font.)

We can elaborate cytokine profiles for Type I subsets with this Table.[875]

	NKT1	**NKT2**	**NKT17**
Express	*T-box 21* (T-bet) [lower TCR expression than NKT2 or NKT17]	*Gata-binding protein 3* (GATA3) [high TCR expression]	*retinoic acid receptor-related orphan nuclear receptor gamma* (RORγt) [intermediate expression]
Produce	*interferon gamma* (IFN-γ) and IL-4 (low levels)	IL-4	IL-17 (some IL-4 on stimulation)
Effects	role in tumor rejection	elicits systemic effects; directly conditions CD8 T-cells to become memory cells; stimulates innate immune cells	involved in inflammation, bacterial infection, autoimmunity

[875] Table matter (murine studies) from Kwon and Lee, "Lineage Differentiation Program," Hogquist and Georgiev, "Recent Advances," and Klibi, Amable, and Benlagha, "A Focus."

To this point we have focused on elaborating on the two types of NKT cells and exploring differences between them. But let's revisit the matter of *NKT cell activation*. The illustration below reexamines that part of the initial Table in this section.

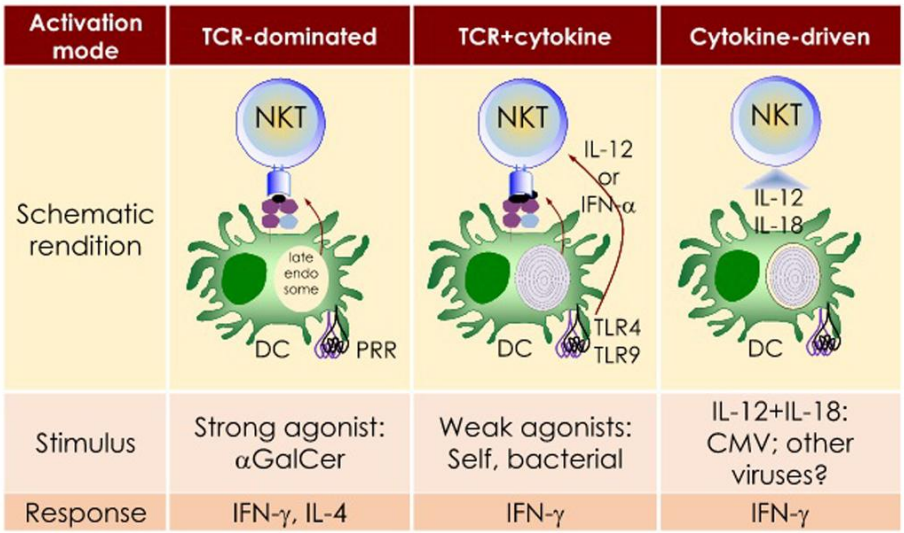


Figure 1 | Three distinct strategies activate mouse NKT cells. Potent NKT cell agonists—such as αGalCer—directly activate NKT cells without the need for a second signal, in a T cell receptor (TCR)-signaling dominated fashion (left panel). Alternatively, microbes containing toll-like receptor (TLR) ligands such as LPS activate NKT cells by inducing IL-12 production by DCs, which amplifies weak responses elicited upon the recognition of CD1d bound with self-glycolipids by the NKTCR. Several endogenous lipid agonists have been identified and characterized (see Table 1). Some microbes such as Sphingomonas capsulata, which are α-Proteobacteria, synthesize α-anomeric glycolipids for their cell walls. These glycolipids, when presented by CD1d, weakly activate NKT cells directly. In the presence of a second signal—generally a pro-inflammatory cytokine such as IL-12—such weak agonists strongly activate NKT cells (middle panel). Intriguingly, NKT cells can be activated solely by cytokines—mainly IL-12— in a TCR-independent manner (right panel). This diagram rendering the different strategies to NKT cells is an adaptation of past reviews (8, 44) and is based on works cited in the text.

The illustration is from Amrendra Kumar, Naveenchandra Suryadevara, Timothy M. Hill, Jelena S. Bezbradica, Luc Van Kaer, and Sebastian Joyce. "Natural Killer T Cells: An Ecological Evolutionary Developmental Biology Perspective." *Frontiers in Immunology* 8 (Dec., 2017) art. 1858 doi: 10.3389/fimmu.2017.01858. Material is from page 5 and made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/ legalcode). Changes have been made to font type used in figure explanation text.

Activated NKT cells participate in important regulatory functions. Once again, the distinctive nature of NKT cells bridging the innate and the adaptive immune

systems can be glimpsed. The following illustration should help.

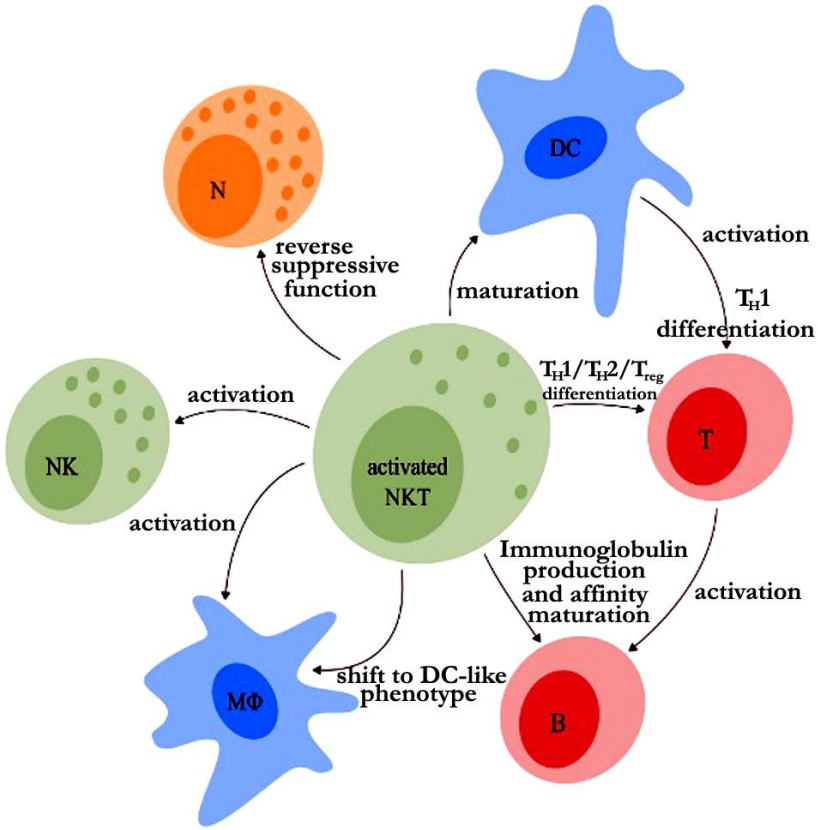


Figure 4 | The regulatory function of activated NKT cells. Upon activation, NKT cells have a major influence on other immune cells. NKT cells can induce full maturation of DC upon which they activate T cells and induce T_H1 differentiation. Activated T cells interact with B cells resulting in their activation, as well as production of immunoglobulins and affinity maturation. The T_{FH} type I NKT cell subset provides cognate help for B cells which promotes the production of immunoglobulins and affinity maturation. In addition, NKT cells can reverse the phenotype of immune suppressive neutrophils. Upon activation, NKT cells are also able to activate NK cells and macrophages and induce a functional shift of monocytes toward a DC-like phenotype. Finally, NKT cells can drive T cell differentiation to either a T_H1-, T_H2-, or T_{reg}-profile, dependent on the NKT subsets involved. Abbreviations: NKT, natural killer T; DCs, dendritic cells; T_H, helper T; T_{FH}, follicular helper T; NK, natural killer; T_{reg}, regulatory T; N, neutrophil; MΦ, macrophage.

The illustration is from Daniëlle Krijgsman, Marianne Hokland, and Peter J. K. Kuppen. "The Role of Natural Killer T Cells in Cancer—A Phenotypical and Functional Approach." *Frontiers in Immunology* 9 (Feb., 2018) art. 367. doi: 10.3389/fimmu.2018.00367. Material is from page 8 and made available by Creative Commons Attribution (CC BY) License (https://creative commons.org/licenses/by/4.0/ legalcode). Changes have been made to font type and size.

Finally, activated NKTs 'crosstalk'—they engage in signaling interactions, using cytokines, toward a common end.[876] This illustration provides a glimpse of this complex context.

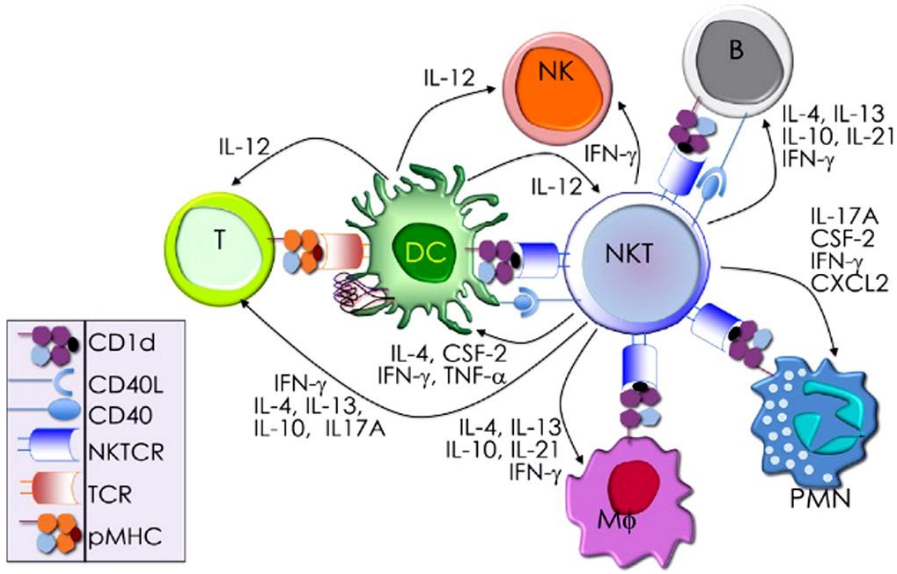


Figure 2 | The immunological effector functions of mouse NKT cells. The interactions between the invariant natural killer T (NKT) cell receptor and its cognate antigen, as well as interactions between costimulatory molecules CD28 and CD40 and their cognate ligands CD80/86 (B7.1/7.2) and CD40L, respectively, activate NKT cells. Activated NKT cells participate in crosstalk with members of the innate and the adaptive immune systems by deploying cytokine and chemokine messengers. Upon activation in vivo, NKT cells rapidly secrete a variety of cytokines and chemokines, which influence the polarization of CD4+ T cells toward T helper (Th)1 or Th2 cells as well as the differentiation of precursor CD8+ T cells to effector lymphocytes, and B cells to antibody-secreting plasma cells. Some of these mediators facilitate the recruitment, activation and differentiation of macrophages and dendritic cells (DCs), which results in the production of interleukin (IL)-12 and possibly other factors. Interleukin (IL)-12, in turn, stimulates NK cells to secrete interferon (IFN)-γ. Thus, activated NKT cells have the potential to enhance as well as temper the immune response. This schematic rendition of NKT cell effector functions is an adaptation of past reviews (6, 8, 44, 70) and is based on works cited in the text.

The illustration is from Amrendra Kumar, Naveenchandra Suryadevara, Timothy M. Hill, Jelena S. Bezbradica, Luc Van Kaer, and Sebastian Joyce. "Natural Killer T Cells: An Ecological Evolutionary Developmental Biology Perspective." *Frontiers in Immunology* 8 (Dec., 2017) art. 1858 doi: 10.3389/fimmu.2017.01858. Material is from page 5 and made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/ legalcode). Changes have been made to font type used in figure explanation text.

[876] For an introduction to 'crosstalk' see Vert and Chory, "Crosstalk in Cellular Signaling."

Measuring Immune Response

In the previous chapter we briefly examined the matter of diagnostic testing to determine if a COVID-19 infection is present. Here we have something different in mind. Our interest is not *present* infection but signs of *previous* infection and how long those signs persist. The reason this matters is that signs of persistence indicate an immune system still activated and ready with antibodies to contest any second invasion of SARS-CoV-2.

Scientists try to measure the human immune system response by using *serology tests*. As the name points out, such tests examine body *serum*—the clear liquid part of blood, absent blood cells and clotting proteins. A serology test looks for antibodies, which indicate the presence of previous infection. Technically, they are examining *seroconversion*, the production of antibodies as marked by their presence in blood. The result is a measurement reported as *antibody titer*—the 'strength of concentration' (*titer*) of antibodies, commonly referred to as the 'level' of antibodies.

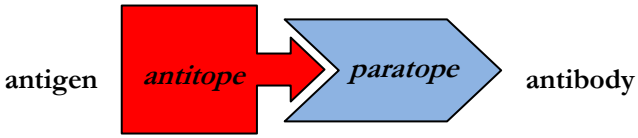
Courtesy of FDA

Serologic assays—that is, 'analyzing tests' (*assays*) examining 'serum' (*serologic*)—measure both the body's production of antibodies against antigenic targets and their functional ability to neutralize SARS-CoV-2. Let's break that down a bit, starting with a simple way of dividing these two interests:

- measuring *binding antibodies* looks for how much of a response is indicated against specific parts of the virus;
- measuring *neutralizing antibodies* looks at how well the body stops infection from happening by binding viral cells before they can attach to host cells.

The first interest concerns *binding antibodies* (also known as 'non-nrutralizing antibodies' (n-NAbs). This kind of testing measures how much antibody response can be detected against one or another part of the SARS-CoV-2 virus that serves as an antigen and provokes an immune response. Three targets on a virus are most common: the spike (S) protein itself, that part of it known as the receptor-binding domain (RBD), or the nucleocapsid (N) protein that protects the viral genome. Unfortunately, while binding antibodies are produced in abundance by the body, they don't prevent viral infection. What they do, however, is alert the immune system to send help. Thus these binding antibodies to not work alone but in concert with other immune system cells. So this first measurement examines *how robust or strong the immune response has been*.

The second interest is measuring *neutralizing antibodies* (Nabs). Researchers Narayanaiah Cheedarla and Luke Elizabeth Hanna explain, "Neutralization is defined as the loss of infectivity that takes place when an antibody molecule binds to a virion and usually does not require the involvement of any other antibody activity."[877] This self-sufficiency immediately contrasts them to the binding antibodies just discussed. An illustration of neutralizing may help.



An antigen has a part recognized by an antibody; that part is termed the *antibody epitope* (or, sometimes, *B-cell antitope*) with its 'internal standard', the antigen's *antitope*, which is a non-functionable peptide generated within the antibody epitope.[878] The corresponding part on the antibody is called the *paratope*, but is also commonly referred to as the antibody's *antigen-binding fragment* (Fab).[879] While it is easy enough to think of the antibody as the neutralizer, sometimes it is specifically *fragments of antibodies* (Fab) or even smaller pieces such as *single-domain antigen-binding recombinant factors* and *natural nanobodies*—so-called 'heavy chain antibodies' (HCABs), which are antibody fragments of just one domain, and thus also referred to as *single-domain antibodies* (sdAbs).[880] No matter what specifically is being referred to, the interest of this measurement is *how effective against infection the immune response has been*.

[877] Cheedarla and Hanna, "Functional and Protective Role of Neutralizing Antibodies," 86.
[878] For more on the matter, see Strehl et al., "Antitopes Define Preferential Proteasomal Cleavage Site Usage."
[879] For more on this subject, see Sela-Culang, Kunik, and Ofran, "The Structural Basis of Antibody-Antigen Recognition."
[880] Cheedarla and Hanna, "Functional and Protective Role of Neutralizing Antibodies," 84.

Chapter 6
The Immune System vs. the Virus

SARS-CoV-2 invades human host cells but the immune system responds to this invasion with a vigorous defense. By way of both review and an overview let us start with an illustration of this invasion and subsequent defense.

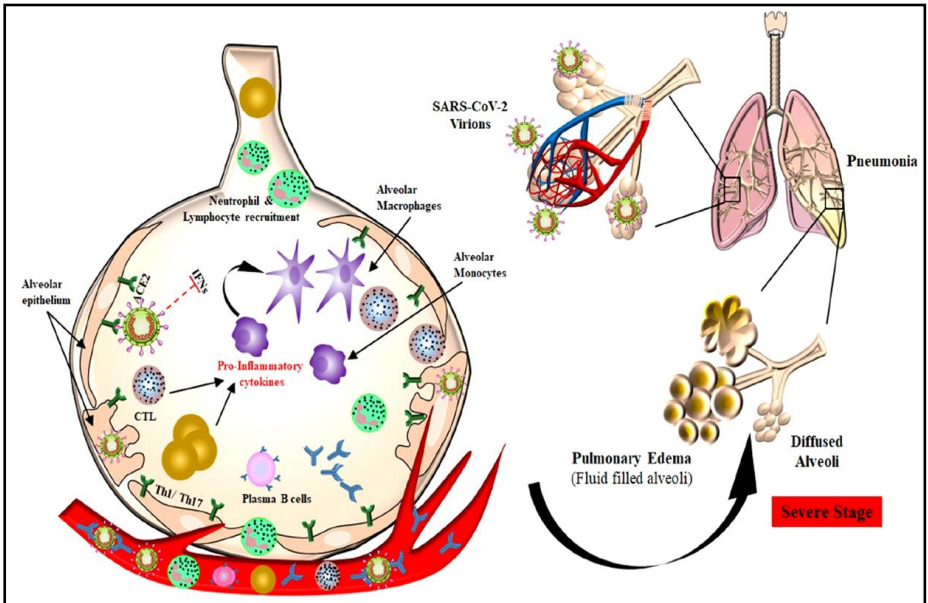


FIGURE 2 | Plausible host immune responses during COVID-19 infection. The SARS-CoV-2 virus infects through the naso-oral route, followed by infection in cells expressing ACE2 receptor in the lung, such as type 2 alveolar cells. These viruses dampen anti-viral IFN responses by evading the innate immune cells as a consequence of unrestrained virus replication. The infiltration of monocytes/macrophages, neutrophils, and several other adaptive immune cells leads to increased pro-inflammatory cytokines. In the helper T cell subset, stimulation of Th1/Th17 cells with viral epitopes may lead to aggravated inflammatory responses. This inflammatory response results in "cytokine storms" that lead to immunopathologies like pulmonary edema and pneumonia. Cytotoxic T cells recruited to the site of infection try to kill virus-infected cells in the lungs. B cells/plasma cells also recognize viral proteins and are activated to produce antibodies specific to SARS-CoV-2, which may help in deactivating viruses and provide systemic immunity in different organs.

The illustration is from Vibhuti Kumar Shah, Priyanka Firmal, Aftab Alam, Dipyaman Ganguly, and Samit Chattopadhyay. "Overview of Immune Response During SARS-CoV-2 Infection: Lessons from the Past." *Frontiers in Immunology* 11 (Aug., 2020) art. 1949. doi: 10.3389/fimmu.2020.01949. Material is from page 6 and is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/ legalcode). Changes have been made to font type in the illustration's explanatory text.

As our opening illustration shows, there is a delicate and complex interplay that goes on in the struggle between SARS-CoV-2 and its unwilling human host. To best utilize this illustration requires drawing upon what has gone before in this volume. This chapter depends on our previous ones examining how the virus attacks the host, including the ways it attempts to avoid immune surveillance and response. This chapter also depends on the examination we just completed about the immune system. Because of what those previous chapters covered we can afford here to be tighter in focus.

The Innate Immune System versus SARS-CoV-2

We have seen the structure and basic function of the innate immune system, which provides a first line of defense against SARS-CoV-2. Immunologists Michael Diamond and Thirumala-Devi Kanneganti neatly summarize the nature of this defense: "Innate immune responses limit viral entry, translation, replication and assembly, help identify and remove infected cells and coordinate and accelerate the development of adaptive immunity."[881] That the innate system is heavily involved in the war against SARS-CoV-2 is evident in the increased prevalence and activation of its immune cells, something especially observed in COVID-19 patients with severe disease.[882]

Working with the adaptive immune system produces a one-two punch combination. First, the innate immune system offers its nonspecific but rapid response aimed at controlling the infection, utilizing both antiviral and proinflammatory molecules, while recruiting, aiding, and sustaining an adaptive immune response, which though nimble takes time to develop.

In the last chapter, we saw that the innate immune system must quickly accomplish three critical defense actions:[883]

1. *Recognize the danger*—a task assigned to pattern recognition receptors (PRRs).
2. *Initiate a cascade of signaling along host pathways*—something accomplished by innate immune signaling pathways.
3. *Produce proteins to inhibit virus replication*—a job well-suited to type I interferons (IFNs).

And, as also noted before, in mounting a defense via these actions the immune system must be willing to recognize when an individual battle has been lost and sacrifice the host cell in the interest of winning the war against the infection and preserving the life of the organism as a whole. Let us see how these actions occur with specific reference to SARS-CoV-2.

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[881] Diamond and Kanneganti, "Innate Immunity," 165.

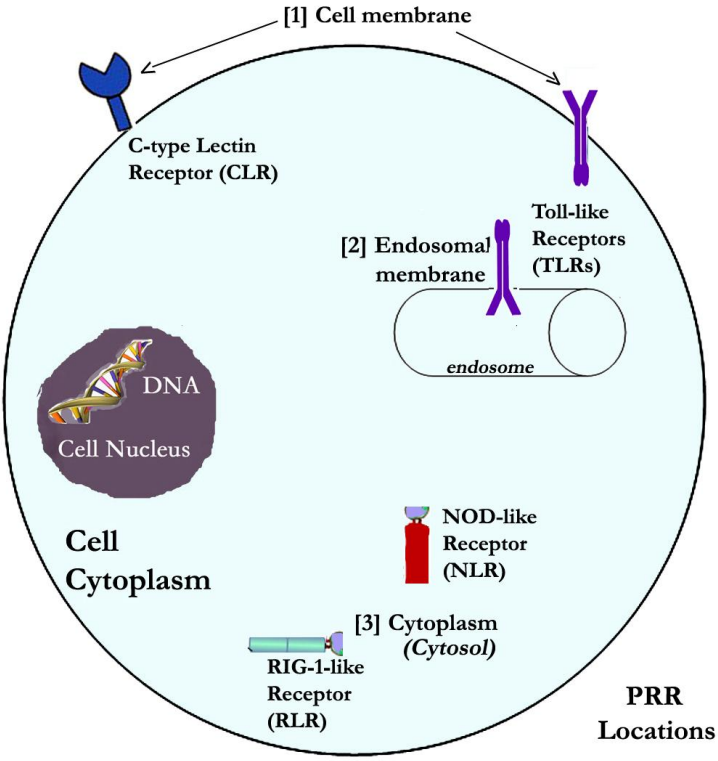
[882] Murgolo et al., "SARS-CoV-2 Tropism," 11.

[883] As noted before, the following points are briefly described in Coleman, "Determining How Coronaviruses Overcome the Interferon and Innate Immune Response," 223–24 (though 'invader' is used instead of 'danger'); elaboration is found in Linares-Fernández et al., "Tailoring mRNA Vaccine."

Defense Activity 1: Recognizing the Danger

Immune defense requires *pattern recognition receptors* (PRRs) capable of recognizing danger. These are found mainly with antigen-presenting cells (APCs) of the innate immune system (e.g., macrophages and dendritic cells). Researchers Prem Kumar and colleagues sum up this first defense as follows: "During surface binding, internalization, endosomal transport, cytosolic uncoating, RNA polymerization, and translation in a host cell, viral lipids, proteins, and RNA are recognized by various pathogen-associated molecular patterns (PAMPs) in the cell membrane, endosomes, and cytoplasm."[884] Put even more simply, from the moment SARS-CoV-2 attaches to a human host cell PRRs start recognizing the danger and they continue to do so throughout the invasion of the virus.

Let's look again at an earlier illustration to remind us of the kinds of PRRs.



Transmembrane PRRs, like other surface-localized immune receptors are *glycoproteins* (molecules with protein and carbohydrate chains). Some PRRs, notably certain *Toll-like receptors* (TLRs) such as TLR2 and TLR4 appear able to recognize extracellular PAMPS in the form of glycoproteins presented by virus envelope features. Viruses like SARS-CoV-2 use glycoproteins on their surface

[884] Kumar et al., "Molecular Recognition," 7.

envelope to facilitate attachment and entry into host cells. SARS-CoV-2 sports a large number of highly glycosylated proteins; the Spike (S) protein of SARS-CoV-2 is a Type I glycoprotein. The *receptor-binding domain* (RBD) of the S protein initiates attachment and viral entry through interaction with host *angiotensin-converting enzyme 2* (ACE2) receptors.[885]

Available evidence shows that PRR responses to SARS-CoV-2 resemble those found for other coronaviruses. We mentioned above TLR2 and TLR4, that recognize PAMPs on extracellular entities. Three *intra*cellular (endosomal/lysosomal) members of this family—TLRs 3, 7, and 8—are among the first to recognize SARS-CoV-2, leading to enhanced interferon (IFN) production. TLR7, in particular, has been identified as playing a central role in response to SARS-CoV-2 infection.[886]

Although differing among cell types, many innate immune system cells express *C-type Lectin receptors* (CLRs). Antigen-presenting cells (APCs) like macrophages and dendritic cells (DCs) express CLRs. The chief purpose of CLRs is to sense non-self glycans (important biomolecules). SARS-CoV-2 presents glycans on its spike (S) protein. Members of the CLR family (DC-SIGN and L-SIGN) recognize this PAMP and interact with it. Unfortunately, they can be co-opted by the virus and become routes of infection.[887]

Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) also activate their signaling pathways in response to SARS-CoV-2. The NLRP subfamily is noted for its role in forming inflammasomes, and NLRP3—an inflammasome sensor—has been implicated from monocyte and lung tissue samples of COVID-19 patients. It appears a number of SARS-CoV-2 PAMPS may trigger NLRP3 inflammasome activation, including the viral nucleocapsid (N) protein, the spike (S) protein, and ORF3a.[888]

Other intracellular PRRs to note include members of the *RIG-1-like receptor* (RLR) family—cytosolic RNA sensors. All three members (RIG-1, MDA5, and LGP-2 mRNA) have been found in the upper respiratory tract (especially in airway epithelium), where the Omicron variant stations its viral base of operations.[889] In humans, both RIG-1 and MDA5 are known to be able to detect coronaviruses.[890]

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[885] On the general matter of glycoproteins, see Rabinovich, van Koooyk, and Cobb, "Glycobiology of Immune Responses." With respect to PRRs, see Pedraza, Betancur, andUrcuqui-Inchima, "Viral Recognition by the Innate Immune System." On SARS-CoV-2, see Duan et al., "The SARS-CoV-2 Spike Glycoprotein"; for even more detail, see Gong et al., "The Glycosylation in SARS-CoV-2 and Its Receptor ACE2."

[886] Shah et al., "Overview of Immune Response," 5; cf. Kumar et al., "Molecular Recognition," 7. See especially Bartolotti et al., "TLR3 and TLR7 RNA Sensor Activation."

[887]Thépaut et al., "DC/L-SIGN Recognition of Spike Glycoprotein."

[888] Diamond and Kanneganti, "Innate Immunity," 166, 169–70, and Zhang, Wang, and Chen, "The Battle between Host and SARS-CoV-2," 4–5.

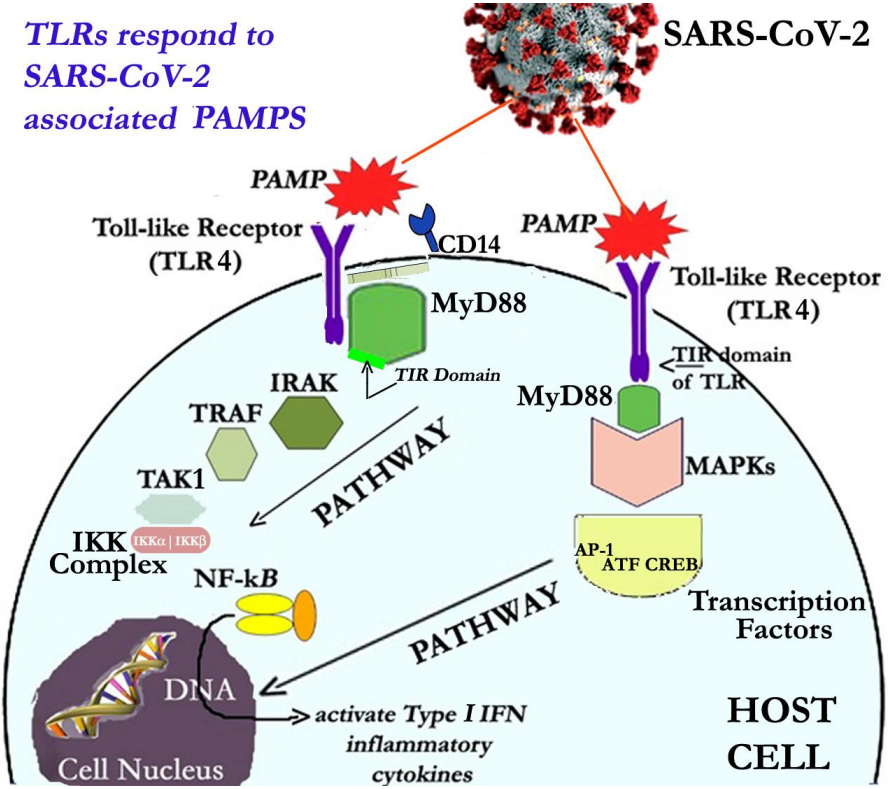
[889] Bogefers et al., "Retinoic Acid-Inducible Gene 1-Like Receptors in the Upper Respiratory Tract."

[890] Rehwinkle and Gack, "RIG-1-Like Receptors," 541 (Table 1); Ricci et al., "Innate Immune Response to SARS-CoV-2 Infection," 3.

Defense Activity 2: Initiate A Cascade of Signaling Along Host Pathways

PRR response gets the ball rolling, which involves the multiple and complex signaling pathways examined last chapter. The molecules PRRs bind to—their *ligands*—trigger these pathways. TLRs use adaptor protein MyD88 with its *toll-interleukin-receptor* (TIR) *domain* to initiate signaling pathways to connect proteins receiving signals from outside the cell to those inside the cell. These pathways tend to modulate transcription factors (TFs), which then lead to changes in cell transcription.[891] In the case of SARS-CoV-2, such TFs include NF-κB, IRF3, and AP-1, which work together to promote type I interferon (IFN-I) production.[892] Signaling activates protein complexes (e.g., *NF-kappaβ* (NF-kβ)) and *Mitogen-activated protein kinases* (MAPKs) to induce inflammatory cytokine genes.[893]

Let's illustrate this situation, using TLR4.



When PRR TLR4 recognizes one specific PAMP (*viral lipopolysaccharide* (LPS)) of SARS-CoV-2, followed by the binding of LPS by the co-receptor pro-

[891] Weidemüller et al., "Transcripton Factors."
[892] Taefehshokr et al., "Covid-19: Perspectives on Innate Immune Evasion," 2.
[893] Kawasaki and Kawai, "Toll-like Receptor Signalling Pathways," 2.

tein CD14, particular signaling pathways (MyD88-dependent and MyD88-independent) are activated. But the same TLR4, when recognizing a different extracellular PAMP of SARS-CoV-2, can activate a different signaling pathway (MAPK)—in each instance leading to secretion of cytokines to control the infection.[894] As the illustration shows, additional players in a TRL pathway to inflammation can include *interleukin-1 receptor-associated kinases* (IRAKs), *TNF receptor associated factors* (TRAFs), *transforming growth factor-β activated kinases* (TAKs), and *IκB kinase* (IKK) *complex* (enzymes IKKα and IKKβ), among others.

It's worth noting that this scenario generally applies to those infected by SARS-CoV-2 who are either asymptomatic or present with mild COVID-19. In cases that are moderate, severe, or critical, the innate immune response has instead deviated from the ideal toward inflammatory damage. Exactly what might tip the immune response from healthy to unhealthy remains unknown.[895]

Other TLRs also can sense SARS-CoV-2. *TLR2* is known to recognize PAMPs on the virus. It is supposed to activate an innate immune response that suppresses excessive inflammation and tissue damage while promoting the integrity of local epithelial barrier function. However, TLR2 and its adaptor MyD88 have been associated with a pathological role contributing to development and severity of COVID-19. *TLR3* and its pathway have been termed a 'major sentinal' against viral infection and may play a significant role against SARS-CoV-2. Mutations in the genes involved in its pathway to producing Type I IFNs have been implicated in some cases of life-threatening COVID-19 pneumonia. *TLR7*, like TLR3 an intracellular PRR, also has been linked to severe COVID-19 cases when mutations in the gene encoding this PRR occur.[896]

C-type Lectin receptors (CLRs) are expressed on antigen-presenting cells (APCs), where they recognize pathogen ligands, to which they attach, internalize the pathogen, and then commonly degrade it via cell lysosomes. Several CLRs (DC-SIGN, L-SIGN, LSECtin, ASGR1, CLEC10A, and TTYH2) have been identified as glycan-dependent binding partners with SARS-CoV-2's S protein. Even though not supporting viral replication, this binding has been associated with inflammatory responses correlating with COVID-19 severity.[897] But, as we've seen, some (DC-SIGN, L-SIGN) may mediate viral entry into host cells.

RIG-1-like receptors (RLRs) RIG-1 and MDA5, and their pathways, have been studied with respect to SARS-CoV-2, though their roles remain substantially obscure. Expression levels of RIG-1 may be especially important during early infection. Reduced levels in lung tissue have been associated with infection and severity of COVID-19. Deficiencies in either RIG-1 or MDA5 enhance viral replication. MDA5 plays a prominent role in activating Type I/III IFN response to SARS-CoV-2.[898]

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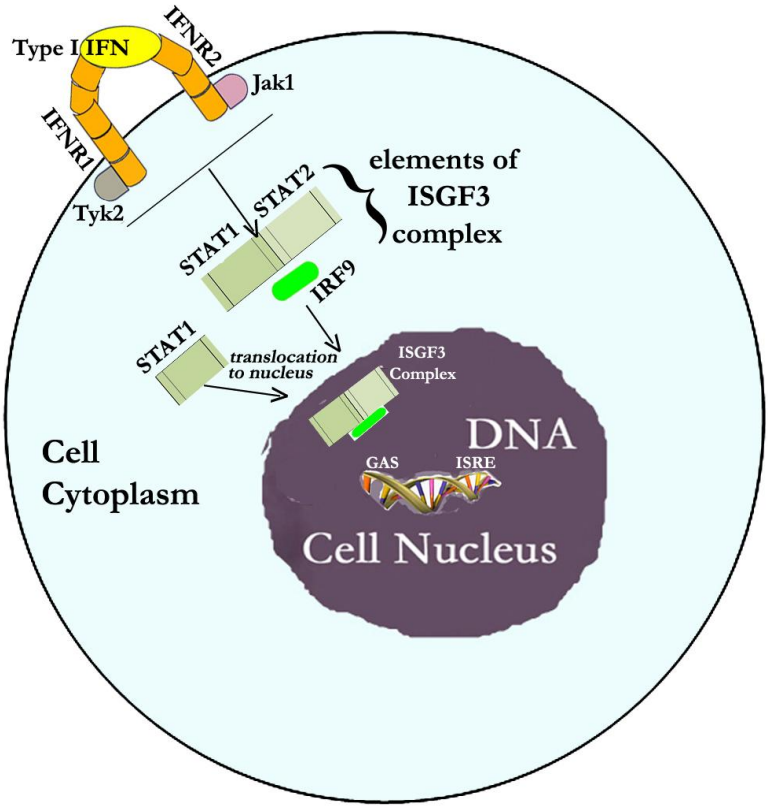
[894] Kumar et al., "Molecular Recognition," 7 (and see p. 8 (Fig. 6)).
[895] Ricci et al., "Innate Immune Response to SARS-CoV-2 Infection," 3.
[896] Zhang, Wang, and Chen, "The Battle between Host and SARS-CoV-2," 3–6.
[897] Zhang, Wang, and Chen, "The Battle between Host and SARS-CoV-2," 6.
[898] Yamada et al., "RIG-1," and Yang et al., "Differential Roles."

Defense Activity 3: Produce Proteins to Inhibit Virus Replication

The third defense activity is an end goal of the first two defense actions. *Host cell production of proteins to inhibit virus replication* is a direct assault on the virus' *raison d'être*. To produce proteins to do cellular work, cells must precisely regulate both transcription and translation. We saw in chapter 1 how these two major steps in protein synthesis occur. But because viruses like SARS-CoV-2 hijack these normal cell processes for their own ends, the human body to survive must form and engage defenses. In essence, if the immune system cannot prevent viral hijacking for viral replication it must at least have a means of inhibiting it.

In this endeavor, human host cells, having identified the viral patterns (PAMPS) that signal a need for action must now *alter translation capacity* (e.g., decrease capacity in order to limit viral protein production).[899] As we saw in the second defensive action, host cells use signaling pathways to activate *Type I interferons*—a kind of cytokine which, recall, 'interferes' with viral replication. We examined the Type I interferon pathway in the last chapter, but the illustration there may be usefully repeated here.



[899] Jaafar and Kieft, "Viral RNA Structure-Based Strategies," 110.

But let's now add to the scene another illustration, one that better shows how this third defense activity is the culmination of a coordinated response. As mentioned elsewhere in this volume, it is easy to lose sight of the forest while examining separate patches of woods or a single tree. The following illustration collects together what we have been talking about and shows all three defense activities as part of one continuous process of defense. (The figure's detailed explanation is continued on the next page.)

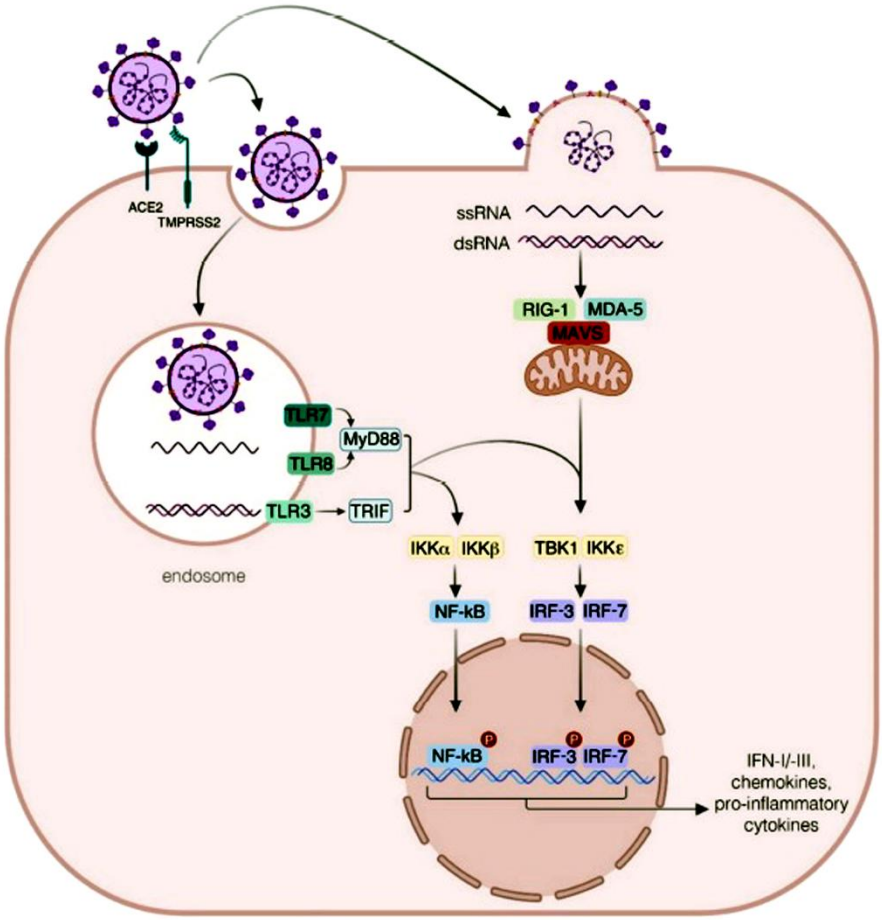


Figure 1. Innate immune response to SARS-CoV-2. The interaction of SARS-CoV-2 with targeted cells requires the recognition of viral spike (S) protein by the angiotensin-converting enzyme (ACE2) and the priming of the S protein by the host cell transmembrane serine protease 2 (TMPRSS2). SARS-CoV-2 can gain access to host cells by means of the endocytic pathway that allows virus localization into the endosomes, where the viral single-stranded (ss) RNA is detected by the endosomal Toll-like receptors (TLR) 7 and 8. Soon after viral RNA recognition, the adapter protein myeloid dif-

ferentiation factor 88 (MyD88), is engaged for the activation and nuclear translocation of transcriptional factors nuclear factor kappa light-chain-enhancer of activated B cells (NF-κB) and interferon regulatory factor-3 (IRF-3) and 7 (IRF-7) for the transcription of genes encoding for proinflammatory cytokines and type I and III interferons (IFNs). In addition, TLR3 can be activated by double stranded (ds) RNA, produced during viral replication, thus contributing via the TRIF pathway to a protective response in SARS-CoV-2 infection. The sensing of replicating virus also occurs by the cytosolic receptor retinoic acid-inducible gene I (RIG-I) and the melanoma differentiation-associated protein (MDA5), which in turn recruits the mitochondrial antiviral signaling protein (MAVS), then leading to the IRF-3 and IRF-7 phosphorylation required for the expression of IFNs. IKKα: I-Kappa-B Kinase Alpha; IKKβ: I-Kappa-B Kinase Beta; IKKε: I-Kappa-B kinase epsilon; TBK1: TANK binding kinase 1; TRIF: TIR domain-containing adapter protein inducing interferon (IFN)-β.

Illustration from Daniela Ricci, Marilena Paola Etna, Fabiana Rizzo, Silvia Sandini, Martina Severa and Eliana Marina Coccia. "Innate Immune Response to SARS-CoV-2 Infection: From Cells to Soluble Mediators." *International Journal of Molecular Sciences* 22 (2021) 7017. https://doi.org/10.3390/ ijms22137017. Material is from page 4 and is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/ legalcode). Changes have been made to font type in the illustration's explanatory text.

Interferons (IFNs)

Clearly the stars of this culminating third defense activity are the interferons (IFNs). Researchers Matthew Galbraith and colleagues remark, "In the context of COVID-19, the role of IFN signaling has been the subject of much study and debate, with both protective and deleterious effects being documented in different experimental systems and clinical settings."[900]

As we have seen, there are three types of Interferons (Types I, II, III); all are involved in the struggle against SARS-CoV-2 and COVID-19.[901] IFNs are the host's major anti-viral weapon, so there is much at stake in the struggle between SARS-CoV-2 and the host immune system with respect to IFN production. If the host prevails, IFNs do what they are intended to do—interfere with viral replication processes. If the virus prevails, IFN production is curtailed or derailed. So let us return to the picture presented by the illustrations above.

Interferons and Pattern Recognition Receptors (PRRs)

IFN production-related PRRs are mostly TLRs, RLRs, and NLRs, [902] all of whom with respect to SARS-CoV-2 we have just briefly reviewed. However, a number of studies have indicated a special prominence to the (Rig-1)-like receptor (RLR) *melanoma differentiation-associated protein 5* (MDA-5). In lung epithelial

[900] Galbraith et al., "Specialized Interferon Action in COVID-19," 10.
[901] For an overview, see Galbraith et al., "Specialized Interferon Action in COVID-19."
[902] Li et al., "Coronavirus Infections and Immune Responses," 427–28.

Calu-3 cells is particularly important for inducing production of both Type I and Type 3 interferons.[903]

IFN-Stimulated Genes (ISGs)

The binding of Type I IFNs to their receptor (IFNAR) leads to activation of the JAK-STAT signaling pathway and promotes expression of *IFN-stimulated genes* (ISGs). Induction of large number of ISGs triggers a global antiviral state. While research of these ISGs with respect to SARS-CoV-2 is ongoing, certain ones to date have been implicated. *Cholesterol 25-hydroxylase* (CH25H) ISG is known to show broad antiviral activity against enveloped viruses, including SARS-CoV-2, which it may frustrate in its membrane fusion activities. But ISGs are active in interfering with SARS-CoV-2 from beginning to end. To date, a number of ISGs (CLEC4D, ELF1, EPSTI1, FAM46C, GBP5, IFIH1, IFITM2, IFITM3, LY6E, REC8, UBD, and XIN2) have been identified as inhibitors of SARS-CoV-2's attempts to use its spike (S) protein for entry into host cells. A number of other ISGs (B4GALT5, DNAJC6, IFIT1, IFIT3, IFIT5, LOC152225, RGSS2, SPATS2L, ZAP, and ZBP1) have been seen to reduce SARS-CoV-2 mRNA levels as well as interfere with viral protein translation or genome synthesis. Finally, ISG *Bone Marrow Stromal Cell Antigen 2* (BST2) may restrict SARS-CoV-2 release, thus limiting spread of the infection.[904]

IFN Type I (Alpha, Beta, Epsilon, Kappa and Omega Subtypes) and SARS-CoV-2

Researchers Cecile King and Jonathan Sprent succinctly capture the importance of Type I IFN (IFN-I) in noting that in conjunction with the products of ISGs, "IFN-I potentiates both the innate and adaptive immune responses to clear viral infections."[905] Type I IFNs are produced early in the immune system response to viral infection and not only work against viral replication but participate in other innate immune system activities. Studies suggest that an initial wave of IFN-β and IFN-α4 then induces through a positive feedback loop increasing waves of Type I IFNs.[906]

Ideally, IFN-I helps regulate the immune system response both indirectly and directly. IFN-I activates antigen-presenting cells (APCs), such as dendritic cells (DCs), which in turn induces natural killer (NK) and T-cell proliferation and differentiation, leading to Type II IFN (IFN-γ) production—these latter consequences the indirect results of Type I IFN activity. At the same time, IFN-I directly acts on cytotoxic T-cells ($CD8^+$) and helper T-cells ($CD4^+$). The former then directly engage infected cells to clear virus while the latter work with B-cells to produce antigen-specific antibodies. But SARS-CoV-2 can reduce

[903] Jouvenet, Goujon, and Banerjee, "Clash of the Titans: Interferons and SARS-CoV-2," 1069; Rebendenne et al., "SARS-CoV-2 Triggers an MDA-5-Dependent Interferon Response."
[904] Zang, Wang, and Chen, "The Battle between Host and SARS-CoV-2," 6–7, referencing Martin-Sancho et al., "Functional Landscape of SARS-CoV-2 Cellular Restriction" (which see).
[905] King and Sprent, "The Dual Nature of Type I Interferons," 312.
[906] Lee and Ashkar, "The Dual Nature of Type I and Type II Interferons," 2.

IFN-I activity, which then leads to dysregulation and diminished effects throughout the chains just described. The result impacts antibody production and facilitates an unhealthy continued production of proinflammatory cytokines.[907] In stark contrast to a mild human coronavirus like HCoV-229E, which induces high levels of Type I IFN expression, SARS-CoV-2 and its more severe coronavirus relatives typically induce a blunted expression of IFN-I.[908]

IFN Type II (Gamma Subtype) and SARS-CoV-2

Type II IFN (IFN-γ) is produced by a number of different cell types, though the most famous producers are the innate immune system's natural killer (NK) cells and the adaptive immune system's $CD4^+$ helper T-cells and $CD8^+$ cytotoxic T-cells. However, antigen-presenting cells (APCs) such as macrophages and dendritic cells secrete IFN-γ and this, as well as that produced by NKs, seems critical in early host defense. APC release of cytokines (especially IL-12 and IL-18) controls IFN-γ production early in the innate system response. These cytokines draw NKs, where IL-12 promotes IFN-γ synthesis. The T-cell production of IFN-γ comes later.[909]

During an antiviral response IFN-γ is predominantly produced by NK cells or innate lymphoid cells (ILCs). IFN-γ plays a robust role in the innate immune response, both directly (e.g., on macrophages and dendritic cells), and indirectly (e.g., inducing intracellular antiviral programs).[910] In COVID-19 its levels are increased ('upregulated'), both systemically and locally in tissue (mucosa). In the latter, for example respiratory mucosa like lung epithelial cells or gastrointestinal mucosa, IFN-γ signaling increases with SARS-CoV-2 infection. But in parts of the human body, such as the colon, IFN-γ is a strong driver of ACE2 expression—thus leading to more of the target receptor preferred by SARS-CoV-2. More of ACE2 means more potential places for the virus to enter and so increases the susceptibility of these cells to infection. In short, rather than successfully curtailing the virus, IFN-γ signaling can promote viral spread. This helps explain the large proportion of COVID-19 patients with gastrointestinal symptoms.[911]

IFN-γ early on emerged as an important indicator of the severity of COVID-19. Higher levels of IFN-γ were associated with a poorer prognosis. Early in COVID-19 infection levels of IFN-γ expectedly rise, but typically are not sustained after 10 days of infection—except in severe cases with an increased mortality rate.[912]

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[907] All this is captured in King and Sprent, "The Dual Nature of Type I Interferons,", 313 (Fig. 1).
[908] Kim and Shin, "Type I and Type III Interferon Responses in SARS-CoV-2 Infection," 753.
[909] Schroder et al., "Interferon-γ: An Overview," 163–64.
[910] Lee and Ashkar, "The Dual Nature of Type I and Type II Interferons," 4–5.
[911] Heuberger et al., "Epithelial Response to IFN-γ Promotes SARS-CoV-2 Infection."
[912] Gadotti et al., "IFN-γ Is an Independent Risk Factor," 3.

Type III IFNs are generally limited in expression (epithelial cells and a subset of myeloid lineage leukocytes). Structurally related to the IL-10 family of cytokines, IFN Type III members include IFN-λ1 (IL-29), IFN-λ2 (IL-28A), IFN-λ3 (IL-28B), and IFN-λ4. Its shared cell surface receptor—the *IFN-lambda receptor complex*—is formed of *IFN Lambda receptor 1* (IFNλR1, aka IL-28Rα) and *interleukin-10 receptor 2* (IFN-10R2). Like Type I IFN, Type III IFN receptor signals via JAK1 and Tyk2, and similarly uses ISGF3 to induce ISG expression.[913]

Both Type I and Type III IFN production is induced to control viral replication happening in primary *human airway epithelia* (HAE) of the respiratory tract when viral activity is high enough to trigger it. Since primary HAE cells are highly susceptible to viral replication, levels high enough to induce a potent response are not difficult to attain. But some research finds the IFN response arrives late after viral exposure. In lung epithelial cells (Calu-3), the main sensor of SARS-CoV-2 appears to be MDA-5 (a RLR PRR). However, even with a robust production of IFNs, viral replication in lung cells may not be controlled if IFN production occurs too tardily. The timing of IFN exposure may be key to controlling viral replication.[914]

IFNs—The Good News and the Bad

As noted above, *timing* and *strength* of IFN response appears to be a critical factor in interfering with SARS-CoV-2 replication. A weak response, or one delayed (or absent) correlates with increased immunopathology and worsened COVID-19 disease.[915] Ideally, rapid IFN production within infected cells transpires soon after infection and limits viral replication within a few days. In such best case scenarios the right innate immune cells do exactly what is needed and to good effect. In other instances, though—and for whatever reason—the viral replication fuels the infection and the immune system effort to compensate leads to exaggerated cytokine and chemokine production. There also may be the recruitment of other immune cell subsets that with the runaway production produces local tissue damage. In a worst case scenario a cytokine storm grows and in severe COVID-19 multiple organ failure and host death may occur.[916]

Infectious disease researchers Daniella Ricci and colleagues remark on the chilling power of SARS-CoV-2 to sometimes win in the struggle against the immune system in this battle over IFN production. "The highly pathogenic potential of SARS-CoV-2," they write, "mainly relies on the peculiar ability to hamper the IFN pathway and, on the other hand, to stimulate an elevated production of proinflammatory chemokines and cytokines. . . ."[917] In this latter instance, a cytokine storm may erupt.

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[913] Kim and Shin, "Type I and Type III Interferon Responses in SARS-CoV-2 Infection," 751.
[914] Rebendenne et al., "SARS-CoV-2 Triggers an MDA-5-Dependent Interferon Response."
[915] Jouvenet, Goujon, and Banerjee, "Clash of the Titans: Interferons and SARS-CoV-2," 1071.
[916] Ricci et al., "Innate Immune Response to SARS-CoV-2 Infection," 4.
[917] Ricci et al., "Innate Immune Response to SARS-CoV-2 Infection," 5.

Interferons (IFNs) are cytokines—'cells' (*cyto-*) that 'change' (*-kine*) the behavior or status of other cells. Although we have focused on IFNs, other cytokines play important roles. Here is a quick overview of some of them.[918]

CYTOKINE	Significant Levels	Clinical Significance (associated with severe disease?)	Prognosis (prognostic value?)
IFN-α	Low IFN-α (inverse association with IL-10)	Yes. Lower levels correlate with higher levels of blood inflammatory cytokines).	Unknown value; peripheral IFN-α not useful as measure of severity
IFN-β	*(generally undetectable)*	Unknown.	(Unknown value)
IFN-γ	High	Yes.	Poor if high levels are prolonged
IFN-λ	Low	Yes.	(Unknown value)
IL-1α	--	No known association.	None known
IL-1β	High	Yes. Associated with cytokine storm.	(Unknown value)
IL-2	High	Yes.	(Unknown value)
IL-3	Low	Yes. (But does not fuel cytokine storm.)	Poor
IL-4	High	Yes. Associated with lung damage	(Unknown value)
IL-5	High	Yes.	(Unknown value)
IL-6	High	Yes. Associated with *Acute Respiratory Distress Syndrome* (ARDS)	Poor
IL-8	High or Low	Levels may increase or decrease with degree of severity (Studies differ)	Poor (worst for critically ill; most useful early on in infection)
IL-10	High (early) Low	Yes.	Poor (useful early predictor)
TGF-β	High	Yes. Associated with cytokine storm.	(Unknown value)
TNF-α	High	Yes. Associated with lung damage.	(Uncertain value as study results vary)

N.B. Table contents should be used with care as studies vary with their findings.

[918] Table content drawn from many sources, including Bénard et al., "Interleukin 3"; Contoli et al., "Blood Interferon-α Levels"; Da Silva et al., "Circulating Type I Interferon Levels and COVID-19"; Ferreira-Gomes et al., "SARS-CoV-2 in Severe COVID-19"; Fouladseresht, et al., Potential Immune Indicators"; Ling et al., "Logitudinal Cytokine Profile"; Mardi et al., "*Interleukin*-1 in COVID-19 Infection"; Merza et al., "Analysis of Cytokines in SARS-CoV-2"; Pituch-Noworolska, "NK Cells in SARS-CoV-2 Infection"; Pons et al., "Cytokine Profiles"; Quartuccio et al., "Interleukin 6"; Trouillet-Assant et al., "Type I IFN."

Cytokine Storms

When SARS-CoV-2 wins against the immune system's intended use of IFNs, devastating results can occur. One is the virus shifting the balance in favor of a proinflammatory cell state that interferes with IFN production. Reduced expression of Type I IFNs together with high levels of proinflammatory cytokines appears in cases of severe COVID-19.[919] Overactivation of the immune system in the form of a massive release of proinflammatory cytokines can lead to a phenomenon already discussed in this volume but worth further examination—*cytokine storms* (aka *hyper-cytokinemia* or *cytokines release syndrome*).[920]

Cytokine storms (cytokines being proteins secreted in inflammation that are important in modulating the inflammation response) result from an inefficient immune response characterized by high levels of inflammatory cytokines (e.g., IL-1, IL-4, IL-6, IL-10, and IFN-γ).[921] But the mechanisms to produce a cytokine storm are manifold. SARS-CoV-2 is able to rapidly activate receptors (PRRs like TLRs and Fc receptors for antibodies), thus incite secretion of proinflammatory cytokines (e.g., IL-6 and *granulocyte-macrophage colony-stimulating factor* (GM-CSF)), which in turn activates other monocytes to produce more cytokines (e.g., TNF-α and more IL-6), all while weak IFN-γ induction may further amplify cytokine production.[922]

In short, the process described in defense activities 1 and 2—PRR activation and signaling along pathways to produce cytokines—draws upon involvement by tissue macrophages, mast cells, endothelial, and epithelial cells. An acute increase in circulating levels of cytokines (e.g., IL-6, IL-1, TNF-α, and interferon) produced by these cells draws more immune cells (macrophages, neutrophils, T-cells) to the site. The result is destabilization of endothelial cell-to-cell interactions, damage of the vascular barrier, capillary damage, and diffuse alveolar damage, so that cytokine storms have been correlated with lung injury, and other features of severe COVID-19 disease such as multi-organ failure.[923]

What begins as a local site of inflammation spreads throughout the body through overproduction of both inflammatory cytokines and chemokines (small proteins that stimulate migration of cells such as white blood cells (leukocytes)), with these being released not just by immune system cells but by other kinds of cells as well (e.g., endothelial cells, smooth muscle cells, and fibroblasts).[924] Put bluntly, such a storm represents a catastrophic failure in the third defense activity—one that jeopardizes the life of the entire host organism.

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[919] Ricci et al., "Innate Immune Response to SARS-CoV-2 Infection," 5–6.
[920] The designation, though popular, is not without controversy. See Sinha, Matthay, and Calfee, "Is a 'Cytokine Storm' Relevant to COVID-19?" Also Knoll, Schultze, and Schulte-Schrepping, "Monocytes and Macrophages in COVID-19," 4.
[921] Bartolotti et al., "TLR3 and TLR7 RNA Sensor Activation," 1.
[922] Hu, Huang, and Yin, "The Cytokine Storm and COVID-19," 251.
[923] Ragab et al., "The COVID-19 Cytokine Storm," 2–3.
[924] Iwasaki et al., "Inflammation Triggered by SARS-CoV-2," 17.

When an Individual Battle is Lost: The Immune System and Cell Death

To try to prevent cytokine storms the immune system utilizes targeted cell death strategies. An infection by SARS-CoV-2 is a war, and not every battle in that war will be won. The host body will lose cells either because the virus' damage is sufficient to kill them or because the immune system destroys them to limit the spread of infection (e.g., apoptosis to block viral replication).

Several times in this volume we have encountered the idea of 'cell death.' It merits a moment for broader consideration. Let's begin with an illustration.

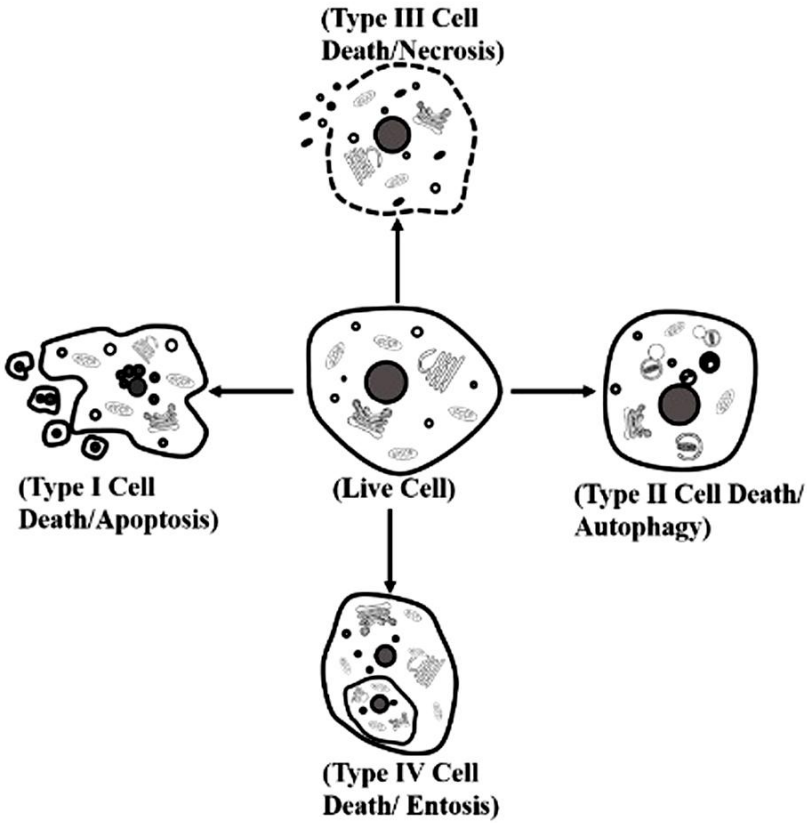

FIGURE 1 | Classification of cell death. Morphologically, cell death is classified into four different forms: type I or apoptosis, type II or autophagy, type III or necrosis, and type IV or entosis.

Illustration from Yun Chen, Yuyun Hua, Xinshuai Li, Ishfaq Muhammad Arsian, Wei Zhang, and Guoliang Meng. "Distinct Types of Cell Death and the Implication in Diabetic Cardiomyopathy." *Frontiers in Pharmacology* 11 (Feb., 2020) art. 42. doi: 10.3389/ fphar.2020.00042. Material is from page 2 and is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/ legalcode). Changes have been made to font type in the illustration's explanatory text.

Of course, the illustration needs elaboration. In general we can make a distinction between four kinds of cell death, each with its own set of characteristics.[925]

Type I ***Apoptosis***	**Type II** ***Autophagy***	**[Oncosis]**	**Type III** ***Necrosis***	**Type IV** ***Entosis***
Normal, natural—'healthy' —*cell suicide.* Fastest form of cell death.	Normal, natural—'healthy' —form of *cell degradation.*	Pre-lethal pathway leading to Necrosis	Cell death—'unhealthy'—associated with cell damage from a pathogen resulting in *cell murder.*	'Cell-in-cell' process after a living cell is engulfed (ingested) by another cell; most die.

Type II Process	Type III Subsets	
Phagocytosis	*Necroptosis*	*Pyroptosis*
'Primary phagocytosis' (or *phagoptosis*) is cell-in-cell engulfment leading to the death of the engulfed cell. Phagocytosis (as *efferocytosis*) is also a way to clean up cellular debris.	Programmed and regulated form of necrosis. Dependent upon either the *serine–threonine kinase receptor-interacting protein 1* (RIP1), or RIP3 for *tumor necrosis factor* (TNF)-induced necroptosis.	Regulated form of necrosis associated with inflammation, activated by the enzyme *caspase-1* (CASP-1) and characterized by pore formation in cell membrane, cell swelling, and membrane disruption.

The above does *not* exhaust kinds of cell death. Other kinds include *alka-liptosis* (a pH-dependent form of programmed cell death), *ferroptosis* (programmed cell death dependent on lipid peroxides), *lysosomal dependent cell death* (LDCD), *neutrophil extracellular trap-related cell death* (NETosis), *oxeiptosis* (a ROS-sensitive, caspase independent, non-inflammatory cell death pathway), and *parthanatos* (a cell death signaling pathway initiated by *Poly-ADP-ribose polymerase-1* (PARP-1)).[926]

Here all we can do is add a few notes on more prominent cell death types.

Cell Death Type I: Apoptosis and SARS-CoV-2

Apoptosis—programmed cell suicide—is a host antiviral defense against viral infection. It can be viewed as part of the third defensive action named earlier—the limiting of virus replication. All human coronaviruses (HCoVs) induce apoptosis, most notably in the respiratory tract; but the same phenomenon has been documented in many cell types elsewhere (e.g., intestinal mucosal cells, kidney tubular cells, neuronal cells, and in lungs, spleen and thyroid).[927]

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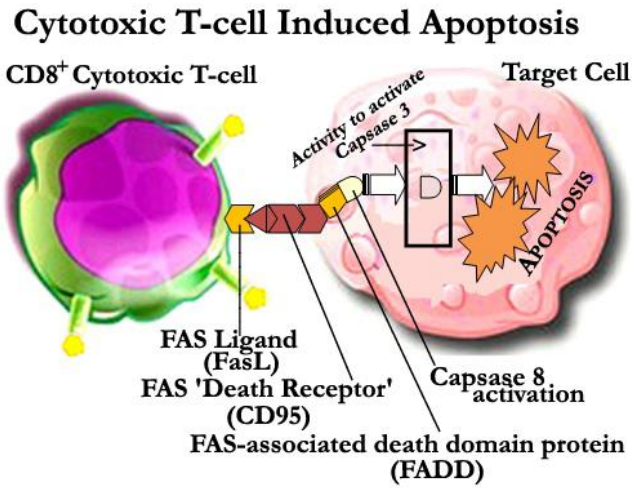
[925] Table draws on a range of sources; see accompanying remarks and citations for some of them.
[926] The list is from Chen et al., "Distinct Types of Cell Death."
[927] Lim et al., "Human Coronaviruses," 5.

It may be worthwhile to reproduce here an illustration of apoptosis.



In the above illustration (more fully explained where it first occurs in this volume), reference is made to caspase 8 activation. SARS-CoV-2 induces such activation triggering apoptosis in lung epithelial cells, at the same time as inflammatory responses in lung tissue and *necroptosis* (a subset of Type III cell death).[928] Apoptosis induced by SARS-CoV-2 has been linked especially to an accessory protein of the virus—ORF3a. However, research suggests that pro-apoptotic activity in this regard is weaker than that seen in SARS-CoV—a finding suggestive of one reason SARS-CoV-2 can be asymptomatic or relatively mild in early stages of infection.[929]

Cell Death Type II: Autophagy as Phagocytosis and SARS-CoV-2

Autophagy (or sometimes *macroautophagy*)—'self' (*auto-*) 'eating' (*-phagy*)—is a biological way of controlling processes of degradation of cellular materials (e.g., proteins and organelles) and is classified as a second major type of programmed cell death. It is a vitally important cellular disposal system, but also provides nutrients and energy as needed. Autophagy works as an immune system process against viruses, as witnessed by viral mechanisms developing to escape it. Among other things, autophagy facilitates the processing and presentation of *major histocompatibility complex class I* (MHC I) *antigen*, which we have seen is important to the work of antigen-presenting cells (APCs). In its role in cell death, autophagy can be activated when apoptosis is compromised.[930]

At various points in this volume we have mentioned an autophagic process known as *phagocytosis*—literally 'eating' (*phago-*) 'cell(s)' (*-cytosis*)—carried out by cells. While all kinds of cells can carry out this process, the group termed *profes-*

[928] Li et al., "SARS-CoV-2 Triggers Inflammatory Responses," 2–3, 5.
[929] Ren et al., "The ORF3a Protein of SARS-CoV-2 Induces Apoptosis in Cells," 883.
[930] Yang and Klionsky, "Eaten Alive: A History of Macroautophagy."

sional phagocytes (or simply *phagocytes*) are most usually discussed. Such cells include neutrophils, monocytes, and macrophages. Phagocytosis includes both . . .

- disposal of waste materials (*efferocytosis*—literally, 'bearing away (dead) cells');
- and cell killing (*phagoptosis*—literally, 'downfall' (*-ptosis*) through 'cell eating' (*phago-* prefix of phagocytosis).[931]

Our particular interest here lies with the latter, termed 'primary phagocytosis.'[932]

Phagoptosis means the doom of a living cell by being eaten by a phagocyte. Put bluntly, phagoptosis is cell cannibalism. It looks like this:

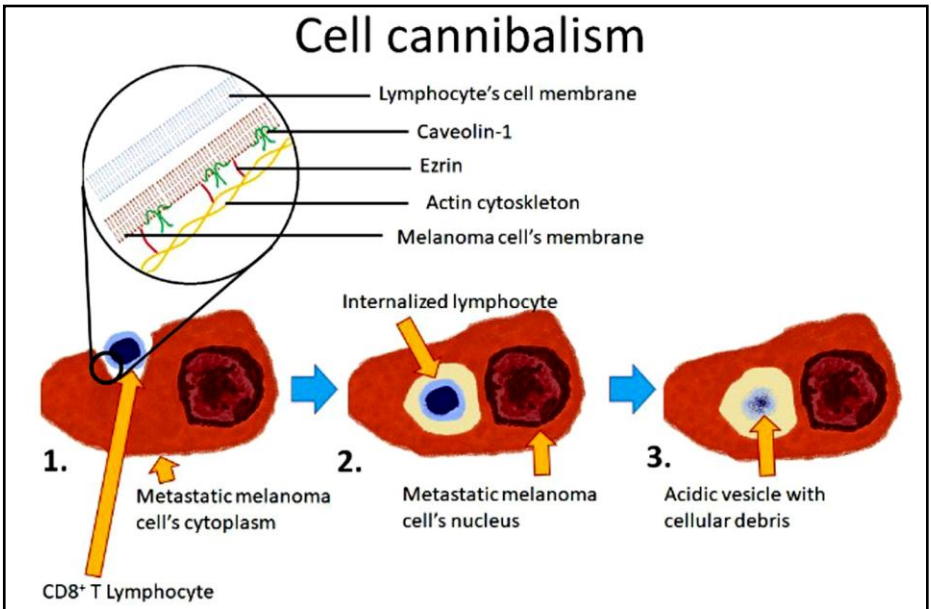


Figure 2. Schematic representation of cell cannibalism of a T lymphocyte by a melanoma cell. 1. The lymphocyte is being actively engulfed by the melanoma cell; 2. The lymphocyte is entirely within the melanoma cell; 3. As lysosomal enzymes are being delivered to the acidified endosome, the lymphocyte is digested.

Illustration from Karol Borensztejn, Pawel Tyrna, Agata M. Gawel, Ireneusz Dziuba, Cezary Wojcik, Lukasz P. Bialy, and Izabela Mlynarczuk-Bialy. "Classification of Cell-in-Cell Structures: Different Phenomena with Similar Appearance." *Cells* 10 (2021) 2569. https://doi.org/10.3390/cells 10102569. Material is from page 5 and is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/ legalcode). Changes have been made to font type in the illustration's explanatory text.

[931] On phagocytosis in general, see Uribe-Querol and Rosales, "Phagocytosis: Our Current Understanding." On how both fit into the larger picture, see Borensztejn et al., "Classification of Cell-in-Cell Structures," esp. p. 3 (Fig. 1). On the subject of efferocytois, see Kojima, Weissman, and Leeper, "The Role of Efferocytosis," 476–77.

[932] On the subject, see Brown and Neher, "Eaten Alive! Cell Death by Primary Phagocytosis."

Phagoptosis with respect to SARS-CoV-2 has been especially discussed with the brain in view. Phagoptosis is attributed to infected microglia in the brain by inducing neurodegradation by phagoptosis of neurons.[933] The results of such damage resemble that associated with traumatic brain injury (TBI) and early on SARS-CoV-2 was suspected of using a similar pathway. It was reported that cytokines IL-1β and IL-18 were found in the plasma of COVID-19 patients and that this is probably related to NLRP3 inflammasome activation through virus proteins such as the envelope (E) protein and ORF3a.[934]

Cell Death Type III: Necrosis as Pyroptosis and SARS-CoV-2

Necroptosis is a programmed, regulated form of necrosis mediated by death receptors. It occurs when the pathway to apoptosis is blocked and requires that caspase-8 (important to apoptosis) be inhibited. It may be an important way for viral clearance in infected cells. It also can induce an inflammatory environment (via release of DAMPS) and recruit immune system help (e.g., immune cells, cytokines, and chemokines).[935] SARS-CoV-2 infection has been found to induce both apoptosis and necroptosis in cell platelets. The presence of both viral S and N proteins suggests platelets are actively removing viral RNA throughout infection.[936] As often seems to be the case, necroptosis is a double-edged sword. On the one hand, it can inhibit viral replication by causing cell death, and yet on the other hand, it can enhance viral dissemination by causing host cell rupture.[937]

Pyroptosis—death by inflammatory processes—is a form of necrosis, unhealthy cell death. In SARS-CoV-2 infection, pyroptosis of monocytes and macrophages occurs and ongoing pyroptosis in COVID-19 patients is especially prominent in severe cases.[938] Pediatric immunologist Judith Lieberman points out, "When cells die by pyroptosis, they release all kinds of inflammatory proteins that cause fever and summon more immune cells to the site"—inducing the cascading signaling associated with cytokine storm—and, "We don't have any way of treating that once it gets started. It's just sort of it's like a little fire. It spreads and explodes and no fire extinguisher is capable of putting it out."[939]

Cell Death Type IV: Entosis and SARS-CoV-2

Entosis—another form of cell-in-cell cannibalism—is known for its role in cancer. However, entosis has been observed in epithelial cells[940] and the in-

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933 Tremblay et al., "Neuropathobiology of COVID-19," 10; Butler et al., "Microglial Phagocytosis of Neurons in Neurodegeneration, and Its Regulation" (esp. 630–31; see 625 (Fig. 2)).
934 Irrera et al., "The Role of NLRP3 Inflammasome," 10–11.
935 Paolini et al., "Cell Death in Coronavirus Infections," 7.
936 Koupenva et al., "SARS-CoV-2 Initiates Programmed Cell Death in Platelets."
937 Yapasert, Khaw-on, and Banjerdpongchai, "Coronavirus Infection-Associated Cell Death," 13.
938 Junquiera et al., "FCγR-mediated SARS-CoV-2 Infection of Monocytes," 1–2.
939 Lieberman quoted in Goodman, "COVID-19 Infections Can Set Off Massive Inflammation."
940 Overholtzer et al., "A Nonapoptotic Cell Death Process."

volvement of SARS-CoV-2 in lung epithelial cells is well-known. But to date entosis does not seem to be a form of cell death associated with COVID-19.

Innate Immune System Cells: Friends *and* Foes

To this point our attention has been on the *processes of defense* against SARS-CoV-2 with a focus on the *weapons of defense* (e.g., PRRs, signaling pathways, and interference with viral replication, including cell death). It is time we back out a bit to see the defenders—the innate immune system cells.

Monocytes, Macrophages, Dendritic Cells

We can summarize some of the engagement of monocytes, macrophages and dendritic cells with SARS-CoV-2 in the following Table.[941]

	Blood Circulating Monocytes	**Dendritic Cells (DCs)**	**Macrophages**
Subsets	*Classical* ($CD14^{+}CD16^{-}$) *Non-classical* ($CD14^{dim}CD16^{+}$) *Intermediate* ($CD14^{+}CD16^{+}$)	*Myeloid DCs* (mDCs) aka *Classical DCs* (cDCs) [cDC1, cDC2] *Plasmacytoid DCs* (pDCs) [$CD303^{+}$]	*Classic* (M1) and *Alternative* (M2), plus proposed . . . *CD169+ Regulatory* (Mregs) *T-cell receptor positive* (TCR^{+}) *Tumor-Associated* (TAM)
Defense Activites 1–2	Express PRRs (TLR2, TLR4, Rig-1, MDA5, CLRs)	cDCs express PRRs (CLR langerin, etc.) pDCs express PRRs (TLR7, TLR9)	Express PRRs (TLR2, TLR4, Rig-1, MDA5, CLRs)
Defense Activity 3	Recruited and activated, they infiltrate inflamed tissues and acquire macrophage and DC-like phenotypes to fulfill effector functions	cDCs act as sentinels; pDCs produce cytokines Type I IFNs); recruit immune cells	Produce cytokines (Type I: subtypes IFN-α1 and IFN-β1 and Type II (IFN-γ) IFNs); phagocytize microorganisms and apoptotic/infected cells
Engagement with Adaptive Immune System	Some become suppressive of T-cells	Both cDCs and pDCs act as antigen-presenting cells (APCs)	As antigen-presenting cells (APCs) they activate adaptive immune system cells

[941] Table compiled from various sources, including Gordon, "Pattern Recognition Receptors"; Labarrere and Kassab, "Pattern Recognition Proteins"; Knoll, Schultze, and Schulte-Schrepping, "Monocytes and Macrophages in COVID-19"; Marongiu et al., "How Dendritic Cells Sense"; Merad et al., "The Dendritic Cell Lineage"; Chávez-Galán et al., "Much More Than M1 and M2."

Monocytes

Monocytes, recall, are produced in bone marrow, circulate for a day or so in the bloodstream, then migrate into tissues where they transform into either macrophages or dendritic cells. Circulating blood monocytes are functionally characterized by three key abilities:

- to phagocytose (ingest and eliminate pathogenic material);
- to produce cytokines; and,
- to present antigen (as antigen-presenting cells (APCs).[942]

Following SARS-CoV-2 infection, peripheral blood monocytes may experience disruption in functions like cytokine production. This leads to an inefficient response to the virus that can enhance pathogenesis and lead to severe COVID-19 and increased mortality.[943]

Most peripheral monocytes are pro-inflammatory at the beginning of an infection. However, blood monocytes can then be 'reprogrammed' from this active state to a suppressive one. Thus, some monocytes (*monocytic myeloid-derived suppressor cells* (mo-MDSCs)) are called *regulatory monocytes* because they are suppressive of T-cells.[944]

Mononuclear Phagocyte (MNP) System

Although all the innate immune system cells may engage SARS-CoV-2, some have received more attention than others. That is why we will begin by looking at macrophages (MPs) and dendritic cells (DCs), which together form the *mononuclear phagocyte* (MNP) *system*. MNP cells, as the name points out, 'phagocytose' pathogens—they ingest dangerous cellular material and eliminate it. Additionally, they act as antigen-presenting cells (APCs) to activate other immune system cells, mediate recruitment of leukocytes (which include the monocytes, granulocytes, B-cells and T-cells), regulate inflammation, and in general initiate and shape immune responses. But excessive activation of MNPs leads to the hyperinflammation described above as 'cytokine storm' and such excess is one important marker of severe COVID-19.[945]

MNPs recognize the threat posed by SARS-CoV-2 using pattern recognition receptors (PPRs), such as TLR2, TLR4, Rig-1 or MDA5, or a variety of CLRs. Alerted MNPs produce cytokines either after direct recognition of viral pathogen-associated molecular patterns (PAMPs) or at the prompting of MNP-recognized damage-associated molecular patterns (DAMPS) released from infected epithelial cells. But while MNPs contest SARS-CoV-2, they also can be infected by the virus, either directly or in the course of phagocytosis of infected alveolar epithelial cells in the lungs.[946]

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[942] Ziegler-Heitbrock, "Blood Monocytes and Their Subsets," 1.
[943] Meidaninikjeh et al. "Monocytes and Macrophages," 3.
[944] Varga and Foell, "Anti-Inflammatory Monocytes," 1.
[945] Knoll, Schultze, and Schulte-Schrepping, "Monocytes and Macrophages in COVID-19," 2.
[946] Knoll, Schultze, and Schulte-Schrepping, "Monocytes and Macrophages in COVID-19," 4.

As has been well publicized from the start of the COVID-19 pandemic, SARS-CoV-2 early establishes its presence in the lungs. There, under optimal conditions (those of homeostatic balance), *macrophages* are the most abundant immune cell type. They are sometimes divided into two groups based on their location. *Interstitial macrophages* (IMs) are found in the tissue in and around the wall of the air sacs (*alveoli*) of the lung, while *alveolar macrophages* (AMs) are in close contact with the type I and II epithelial cells of alveoli (air sacs). The latter offer the first line of defense in the lungs against pathogens.[947]

As mentioned a moment ago, SARS-CoV-2 infects both alveolar epithelial cells and *tissue-resident AMs*. Within these infected AMs, processes initiate recruitment of memory T-cells to the alveolar space; activated there, these T-cells release IFN-γ, which in turn activates infected tissue-resident AMs, which die, but recruit more AMs—this time *monocyte-derived AMs* (MDMs). Their infection contributes to the perpetuation of an inflammatory signalling loop with T-cells. Moreover, it has been suggested, these infected AMs may act like Trojan horses—a supposedly welcome entity that instead conveys the enemy into adjacent healthy lung tissue, which then becomes infected and furthers the spread of SARS-CoV-2.[948]

The dysregulated cytokine response in SARS-CoV-2-challenged MDMs and AMs displays as an attenuated interferon (IFN) response. Macrophage transcript expression of Type I (IFN-α1 and IFN-β1) and Type II (IFN-γ) IFNs increases early at exposure to the virus but then drops later—sometimes to below normal levels of expression. It has been suggested this attenuated response offers a mechanism for slow viral clearance—up to two weeks after infection.[949]

Finally, a study reported in April, 2022, found that about 8% of lung macrophages and 10% of blood monocytes in COVID-19 had been infected by SARS-CoV-2. However, this research also found that although replication begins in monocytes it is aborted as the infected cells experience inflammatory cell death (*pyroptosis*), release inflammatory cytokines, and activate inflammasomes (NLRP3 and AIM2). Tissue-resident macrophages activate inflammasomes, too. In short, it appears that while monocytes and macrophages stop viral replication (defense activity 3), they also promote a systemic inflammation that contributes to COVID-19 pathogenesis.[950]

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[947] Hu and Christman, "Editorial: Alveolar Macrophages," 1.

[948] Grant et al., "Circuits between Infected Macrophages," 640. On the differences between tissue-resident and monocyte-derived macrophages, see Lahmar et al., "Tissue-Resident Versus Monocyte-Derived Macrophages."

[949] Abdelmoaty, Mai. M., et al., "Defining the Innate Immune Responses," 12.

[950] Junquiera et al., "FCγR-mediated SARS-CoV-2 Infection of Monocytes" (see esp. pp. 1–4).

Dendritic cells (DCs) also are important warriors in the war against SARS-CoV-2. The following Table offers some introductory information for both types of DCs and the DC subtypes.[951]

DENDRITIC CELLS ***Types***	**Myeloid DCs (mDCs)** *aka* **Classical DCs (cDCs)**	**Plasmacytoid DCs (pDCs)**
Subsets	**cDC1** (cDC141^{hi}) **cDC2** (cDC1^{+}) **DC3** (cDC1c^{+}CD14^{+})	--
Location	**cDC1** (lymphoid and non-lymphoid organs) **cDC2** (lymphoid organs) **DC3s** (uncertain)	Lymphoid tissues (lymph nodes, Peyer's patches, spleen, thymus, tonsils)
PRRs	**cDC1**: CLRs (CLEC9A) DC-SIGN TLR2, TLR4 **cDC2**—large repertoire **DC3**: TLRs	TLRs (TLR 4, TLR 7, TLR9), CLRs, RLRs (RIG-1, MDA5, LGP2)
Important Function(s)	Acts as immune system sentinels **cDC1s** produce Type I and Type III IFNs and IL-12; can activate NK and Th1 cells **cDC2s** produce large amounts of IL-12, plus other cytokines; can activate Th1, Th2, Th17, and CD8^{+} cells; more abundant in blood and peripheral tissues **DC3s** activate Th1, Th17; change CD8^{+} T-cells into CD8^{+}CD103^{+} *tissue-resident memory* (TRM) T-cells	Chief function is recognition of PAMPs and response to them Primary producer of Type I interferons (IFN-1, IL-6, TNF-α) Link innate and adaptive immune systems with functions devoted to each—e.g., activation of cDCs, support of T-cell survival, and B-cell differentiation

[951] Information in Table drawn from various sources, including Niles et al., "Macrophages and Dendritic Cells"; Pérez-Gómez et. al., "Dendritic Cell Deficiencies"; Ye et al., "Plasmacytoid Dendritic Cell Biology"; Merad et al., "The Dendritic Cell Lineage"; Lundberg et al., "Human Blood Dendritic Cell Subsets"; Chistiakov et al., "Plasmacytoid Dendritic Cells"; Satoh et al., "Human DC3."

There are two principal types of DCs—so-called *classical* (cDCs), or *myeloid dendritic cells* (mDCs) arising from bone marrow tissue, and *plasmacytoid dendritic cells* (pDCs), derived from either myeloid or lymphoid progenitors, and characterized by secreting high levels of Type I interferons.

Myeloid DCs (mDCs) are perhaps today more commonly known as *classical DCs* (cDCs). They are 1–5% of tissue cells (depending on organ). These DCs act as sentinels and respond to stimuli in their environment by undergoing phenotypic changes to become the subset DC needed.[952] Subsets are typically delineated as cDC1 and cDC2, but other specifications are found in the literature (e.g., $CD141^+$ and $CD1c^+$; $CD16^+$ DCs are a unique subset functioning as APCs[953]). Both cDC subsets originate from the same precursor ($CD123^+$).[954] More recently a cDC3 subset (as a population of cDC2) was proposed, and it has gained traction (as simply DC3, not necessarily as a subset of cDC2).[955]

The cDC1 subset is found in primary (e.g., thymus) and secondary lymphoid organs (e.g., lymph nodes), and non-lymphoid organs (e.g., lung, skin, intestine, liver).[956] The cDC1s are able to take up, process, and present (*antigen cross-presentation*) to $CD8^+$ T-cells.[957] In COVID-19 patients, cDC subsets are reduced in blood. Activated cDC2, preferentially recruited over cDC1, has been observed to accumulate in the lungs of those critically ill. It seems DC3, as well as monocytes, are recruited from the blood to the lungs.[958]

Plasmacytoid DCs (pDCs) constitute a miniscule amount (0.1–0.5%) of human *peripheral blood mononuclear cells* (PBMCs), yet their production of Type I IFNs accounts for most of the IFN-I detectable in blood after viral infection.[959] Against SARS-CoV-2, their numbers are reduced and their functionality impaired.[960]

Earlier, in discussing the mononuclear phagocyte (MNP) system, we saw that while MNPs contest SARS-CoV-2, they also can be infected by the virus, either directly or in the course of phagocytosis of infected alveolar epithelial cells in the lungs. After SARS-CoV-2 succeeds in gaining entry into a host it begins replication, which induces cell death by pyroptosis. Such cell death results in PAMPs, DAMPs, and cytokines being released. Among those drawn to the site are DCs. There, it has been proposed, DCs may use DC-SIGN as a receptor and via it the virus 'hides' in DCs while impairing their functioning.[961]

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952 Merad et al., "The Dendritic Cell Lineage."
953 On $CD16^+$ DCs, see Fromm et al., "CD16 Dendritic Cells."
954 Marongiu et al., "How Dendritic Cells Sense and Respond to Viral Infections."
955 Villar and Segura, "The More, the Merrier: DC3s Join the Human Dendritic Family"; Soto et al., "The Role of Dendritic Cells," 7.
956 Collin and Bigley, "Human Dendritic Cell Subsets."
957 Soto et al., "The Role of Dendritic Cells," 4.
958 Winheim et al., "Impaired Function and Delayed Regeneration of Dendritic Cells," 2, 17.
959 Ye et al., "Plasmacytoid Dendritic Cell Biology," 1; Zhou et al., "Acute SARS-CoV-2 Infection," 866.
960 Winheim et al., "Impaired Function and Delayed Regeneration of Dendritic Cells," 3.
961 Campana et al., "Dendritic Cells and SARS-CoV-2 Infection," 8–16 (esp. 11–14).

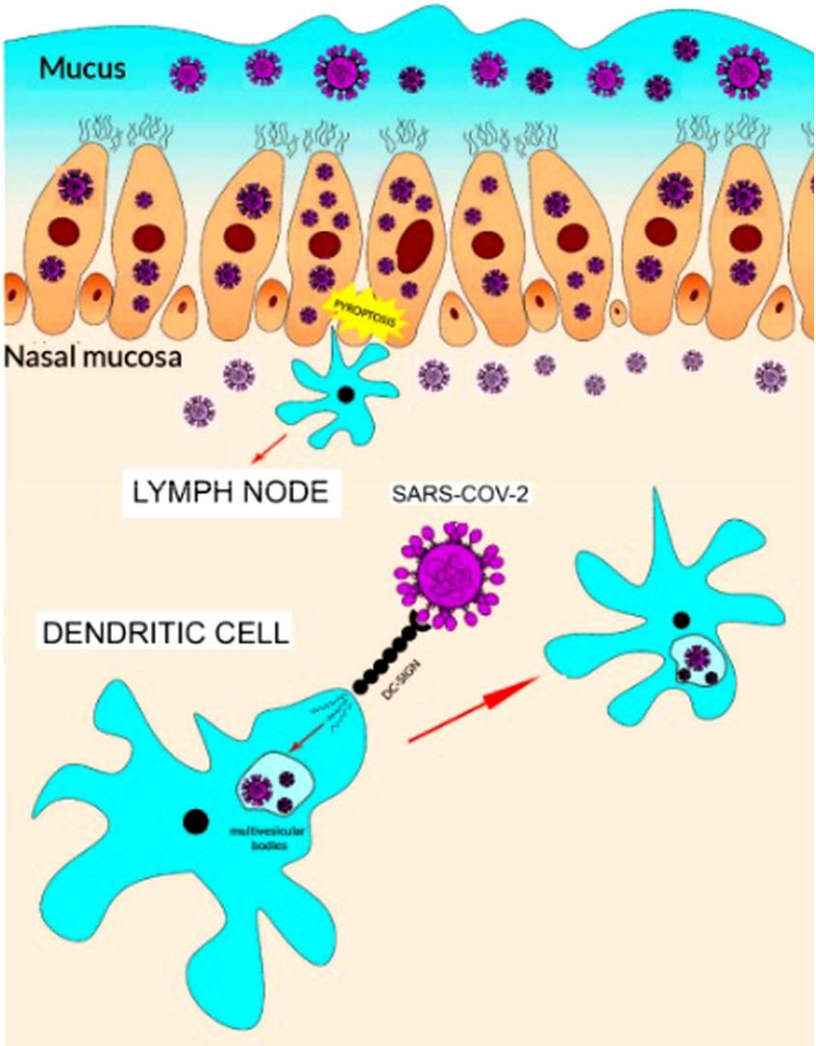

Figure 3. Scheme showing the putative dendritic cells involvement in SARS-CoV-2 entry through nasal epithelial cells. The entry of SARS-CoV-2 through epithelial cells is followed by pyroptosis and activation of dendritic cells in a manner similar with that reported in Figure 2. Particularly, the interaction between DC-SIGN and the virus is shown. The binding (and DAMP and PAMP interaction that is not shown) could cause the virus to spread from the nasal epithelial cells to the rest of human body.

Illustration from Pasquale Campana, Valentina Parisi, Dario Leosco, Debora Bencivenga, Fulvio Della Ragione, and Adriana Borriello. "Dendritic Cells and SARS-CoV-2 Infection: Still an Unclarified Connection." *Cells* 9 (2020) 2046. doi:10.3390/cells9092046. Material is from page 8 and is made available by Creative Commons Attribution (CC BY) License (https:// creativecommons.org/licenses/by/4.0/ legalcode). Changes have been made to font type in the illustration's explanatory text.

The opportunism of SARS-CoV-2 in what is shown by the illustration helps explain why innate immune system cells can be both friend *and* foe to the host. The processes—such as pyroptotic cell death—intended to *contain* infection may in some instances be used by the virus to *spread* infection. Thus, DCs recruited to the place where pyroptosis occurs recognize SARS-CoV-2-associated PAMPs and DAMPs through their PRRs, which then initiate signaling pathways, and this results in Type I IFN production. DCs are also ingesting, transporting, processing, and presenting virus-related antigen to T-cells. (DCs are so adept as professional antigen-presenting cells (APCs) that it has been estimated that a single one can activate anywhere from 100–3,000 T-cells.[962]) All this is expected and can be quite useful in defense against the virus. But it may be that SARS-CoV-2 can bind to the DC surface (using DC-SIGN), thus gaining a free ride to infect cells DCs encounter—a process called *trans-infection*. And, of course, as already noted (and shown in the illustration), SARS-CoV-2 may gain entry into DCs and thus evade immune surveillance by literally wearing the cloak of the body's defender.[963]

With both kinds of DCs (mDCs and pDCs), lower levels have become associated with poorer prognosis for COVID-19 outcomes. Some research has shown that infection by SARS-CoV-2 producing more severe COVID-19 is associated with a decrease of DCs in peripheral blood. This decrease appears linked to some DCs (e.g., $CD1c^+$ subset) migrating to sites of inflammation, like the lungs. With pDCs, critical cases of COVID-19 display highly impaired Type I IFN-response—a deficiency linked both to high viral load of SARS-CoV-2 and an aggravated inflammatory response. Remember, pDCs show 1000-fold production of Type I IFNs compared to other immune cell types (which is why they are known as 'the IFN factory of the immune system.'[964]). Thus, pronounced impairment of pDC function can be expected to end in very deleterious consequences. Moreover, the consequences can be long term; deficits observed in both mDCs ($CD1c^+$ subset) and pDCs have been reported to persist beyond seven months past infection. [965]

The diminished production of Type I interferons (and Type III) is not the only consequence possible from SARS-CoV-2 infection. The decreased number and functionality in DCs also can contribute to delayed T-cell responses by reducing the induction of these adaptive immune system cells.[966]

Much as has been the case with macrophages, the role of DCs in the production of pro-inflammatory cytokines that lead to cytokine storm after infection by SARS-CoV-2 remains a matter of some debate.[967] What is not up for debate is that severe COVID-19 hinges in part on DC performance.

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[962] Campana et al., "Dendritic Cells and SARS-CoV-2 Infection," 10.
[963] Chang et al., "Depletion and Dysfunction of Dendritic Cells." See, esp., p. 3 (Fig. 1).
[964] Wack, "Monocyte and Dendritic Cell Defects in COVID-19," 445.
[965] Pérez-Gómez et. al., "Dendritic Cell Deficiencies," 2128, 2132.
[966] Zhou et al., "Acute SARS-CoV-2 Infection," 870–71.
[967] Niles et al., "Macrophages and Dendritic Cells," 9.

Natural killer (NK) *cells* live up to their name by their principal function of being cytotoxic of dangerous cells (e.g., those damaged by infection or tumorous). They are an important producer of cytokines such as IFN-γ. NKs are commonly divided into those typically formed in bone marrow and then released into circulation—*conventional NK cells* (cNKs)—and nonconventional *tissue-resident NK cells* (trNKs). The former are noted for their cytotoxicity; the latter's degree of cytotoxicity depends on their location (e.g., in liver tissue it is low, but it is notable in lung tissue). They chiefly kill dangerous cells by direct lysis (i.e. rupturing the cell membrane to destroy its integrity and result in its disintegration).[968]

With SARS-CoV-2 infection, cNKs are recruited to where the infection is occurring. Most notably, this is the lungs. The spike (S) protein, through its extracellular subunit 1 (S1), is able to induce directional movement (*chemotaxis*) and activation of NKs, yet in lung tissue the intracellular expression of the S1 protein both reduces the activation of NK cells and their ability to degranulate (which is the mechanism by which they effect cytotoxicity).[969]

It appears that the virus S1 protein accomplishes this effect by binding to *HLA class I histocompatibility antigen, alpha chain E* (HLA-E), a molecule on NKs that serves as a binding site ('ligand') for the *CD94/NK group 2 member A* (NKG2A) r*eceptor* of NKs—an inhibitory receptor. Crosstalk between HLA-E and NKG2A/CD94 induces a higher surface level of HLA-E molecules at the same time as a prevalent expression of NKG2A. One study found the percentage of NKG2A-positive cells increased from the 16% to 80% in lung epithelial cells. Then an activated *HLA-E/NKG2A pathway* reduces NK degranulation and thus severely affects NK cytotoxicity. Recovered COVID-19 patients show normal NK counts with reduced NKG2A levels, suggesting that reversal of the viral influence has occurred.[970]

NK levels are associated with different degrees of disease severity in COVID-19. Lower levels are found with critically ill patients, and in such patients cNKs show low levels of IFN-γ, TNF, IL-2 and granzyme production. The resulting reduced cytotoxicity means they become ineffective against the virus, likely due to SARS-CoV-2's use of mechanisms to inhibit them. It has been speculated that the lower number of cNKs may be the result of redistribution to infected tissues even though these cell's are exhausted and die.[971]

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[968] Pituch-Noworolska, "NK Cells in SARS-CoV-2 Infection," 4–5.

[969] Bartolotti et al., "SARS-CoV-2 Spike 1 Protein Controls Natural Killer Cell," 11.

[970] Ahmed, Jo, and Lee, "Can Natural Killer Cells Be a Principal Player in Anti-SARS-CoV-2 Immunity?" 3–4, and Bartolotti et al., "SARS-CoV-2 Spike 1 Protein Controls Natural Killer Cell," 12. Also see Lee et al., "HLA-E Is a Major Ligand."

[971] Pituch-Noworolska, "NK Cells in SARS-CoV-2 Infection," 6.

Innate immune system *granulocytes*, the most common white blood cells and bearers of packets of very fine particles ('granules') include basophils, eosinophils, neutrophils, and mast cells. We can summarize some of their engagement with SARS-CoV-2 in the following Table.[972]

	Basophils	**Eosinophils**	**Neutrophils**	**Mast Cells**
Defense Activites 1–2	Express PRRs (TLR2, TLR4)	Express PRRs (TLR1, TLR2, TLR3, TLR7, TLR9, TLR10)	Express PRRs (TLR8)	Express PRRs (TLR3, TLR9, NOD, RIG-1)
Defense Activity 3	Release cytokines (IL-4, IL-13), and chemokines (CXCL8/IL-8 and CCL3/MIP-1α)	Synthesize, store, release variety of cytokines and chemokines; upon exposure to virus, degranulate and release proinflammatory Type I cytokines (e.g., IL-12)	Produce cytokines and chemokines (CXCL-2 and CXCL-8); neutrophil extra-cellular traps (NETs)	Release broad spectrum of cytokines and chemokines
Engagement with Adaptive Immune System	Express high affinity receptor (FcεRI) for IgE; act synergistically with DCs as APCs to $CD4^+$ T-cells; induce TH2 via release of IL-4	Serve as antigen-presenting cells (APCs) to T-cells	Promote TH17 activity	Express high affinity receptor (FcεRI) for IgE

Basophils (and eosinophils) increase from the acute phase of COVID-19 infection to recovery. They bind antigen on their surface and increase humoral immune responses. In general, basophils may enhance the adaptive immune response that may be suppressed by a hyperinflammatory reaction during an infection's acute stage. Their depletion may impair the efficacy of antibody IgE.[973]

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[972] Table compiled from various sources, e.g., Murdaca et al., "Basophils and Mast Cells"; Eid and Borish, "Eosinophils in Antiviral Immunity"; Pala and Pistis, "Anti-IL5 Drugs in COVID-19"; Reusch et al., "Neutrophils in COVID-19;" Cavalcante-Silva et al., "Neutrophils and COVID-19"; and,Thomas and Schroder, "Pattern Recognition Receptor Function in Neutrophils."

[973] Rodriguez et al., "Systems-Level Immunomonitoring," 9, 11; Murdaca et al., "Basophils and Mast Cells," 5.

Eosinophils are known to mount a robust defense against viruses. They may migrate to lungs where their *lysis* (cell membrane rupture) releases granules and produces damage-associated molecular patterns (DAMPs) that lead through signaling to the recruitment of phagocytes.[974] Among granules released are preformed *eosinophil cationic protein* (ECP)—a cytotoxic antiviral agent—and preformed cytokines (IL-2, -3, -4, -5, -6, -10, -12, and -13, IFN-γ, TNF-α, NGF, GM-CSF, stem cell factor, TGF-α, etc.).[975] They also release *eosinophil-derived neurotoxin* (EDN), a protein capable of degrading single-strand RNA substrates, which is elevated in SARS-CoV-2 infection.[976] Eosinophils may be a valuable biomarker of severity of COVID-19; *eosinopenia*—a significant reduction in granulocytes indicative of severe infection—has been found to be present in a majority of patients admitted to hospital or dying.[977] This may be because uncontrolled phagocyte activation leads to cytokine storm.[978]

Neutrophils are recruited to the site of inflammation in the lungs but too much of a presumably good thing can evolve into deleterious consequences. Their increase upon SARS-CoV-2 infection (e.g., in nasopharyngeal epithelium, then more distal parts of lung) can lead to intensification of inflammation and cytokine storm. Severity of COVID-19 is associated both with increased levels of immature neutrophils and with particular kinds of mature neutrophils (e.g., *myeloid derived suppressor cell* (MDSC)-*like* neutrophils and *programmed death-ligand 1 positive* (PD-L1$^+$) neutrophils), and especially with an elevated neutrophil to lymphocyte ratio (NLR).[979]

Mast cells (MCs) regulate inflammatory responses via release of mediators (β-tryptase, chymase, and CPA-3) that contribute a number of biological responses, but which in amplified release contribute to pathogenesis. MCs increase in number in lung tissue and may play a key part in triggering systemic cytokine storm because of viral manipulation (i.e., activation via PRR TLRs). However, it has been suggested this result may not be due to direct infection but instead that it is the secondary activation of cells like MCs and eosinophils via PRRs that contributes to inflammatory cytokine release.[980] One of the substances released by MCs is histamine—well known to allergy sufferers—involved in expressing IL-6 and IL-8, which can amplify lung inflammation.[981]

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[974] Pala and Pistis, "Anti-IL5 Drugs in COVID-19 Patients," 3.
[975] Muniz, Weller, and Neves, "Eosinophil Crystalloid Granules."
[976] Gebremeskel et al., "Mast Cell and Eosinophil Activation," 3.
[977] Lindsley, Schwartz, and Rosenberg, "Eosinophil Responses," 2–3; cf. Pala and Pistis, "Anti-IL5 Drugs in COVID-19 Patients," 4. On reduction of eosinophils associated with COVID-19, also see Xie et al., "The Role of Peripheral Blood Eosinophil Counts in COVID-19 Patients."
[978] Pala and Pistis, "Anti-IL5 Drugs in COVID-19 Patients," 3–4.
[979] Niedźwiedzka-Rystwe et al., "Interplay between Neutrophils, NETs, and T-Cells in SARS-CoV-2 Infection," 6; also Reusch et al., "Neutrophils in COVID-19," and Hazeldine and Lord, "Neutrophils and COVID-19."
[980] Gebremeskel et al., "Mast Cell and Eosinophil Activation," 7–9; Murdaca et al., "Basophils and Mast Cells," 6–8.
[981] Conti et al., "Mast Cells Activated by SARS-CoV-2 Release Histamine."

Special Note: Innate Humoral Immune Response

Near the beginning of the previous chapter, in outlining a structural view of the immune system, four parts were named, with the first three being cell-based but varying in size of the system (organs, tissues, cells). The fourth part concerned macromolecules in extracellular fluid. This, it was indicated, concerns the humoral immune response.

Not uncommonly when the humoral immune system is mentioned the mentioner really has in mind only an adaptive immune system aspect—the work of antibodies. In this perspective, humoral immunity refers to an immune response dependent on antibodies while cell-mediated immunity refers to an immune response absent antibodies.

Such usage is not what is in mind here. The *humoral immune response* in the broadest sense refers to responses involving macromolecules found in extracellular 'fluid' (Latin noun *humor* and adjective *humoralis*). These fluids include the plasma of the circulatory system, lymph of the lymphatic system, and the fluids of the interstitium (the space between internal structures and such structural barriers as cell membranes, or skin). Antibodies may be the most famous immune macromolecules found in fluids, but they are not the only ones.

So the picture we need is of humoral immunity as a response system bridging innate and adaptive immune systems—and thus fitting into our presentation here, between innate and adaptive immunity. A Table overview will help.[982]

INNATE Immune System	**ADAPTIVE Immune System**
Humoral	***Immunity***
natural antibodies (natural IgM, IgA, IgE) pattern recognition molecules (PRMs) (collectins, ficolins, pentraxins) serine protease cascades (complement and contact systems)	immune antibodies (and attendant processes)

We shall look at immune antibodies with respect to SARS-CoV-2 in the next part of the chapter as we focus on the adaptive immune system. Here we shall briefly consider only the *innate* immune arm of the humoral immune response.

[982] Table accepts Shishido et al., "Humoral Innate Immune Response and Disease" for architecture of the innate side.

Natural Antibodies (NAbs)

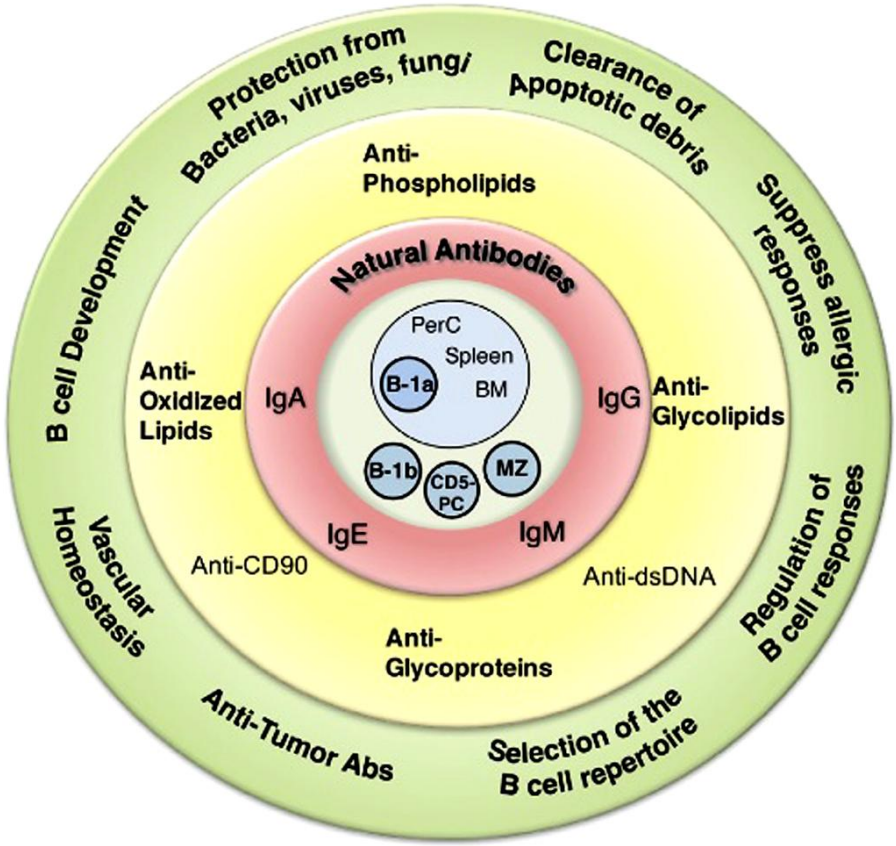

Figure 1 | Overview of natural antibodies' (NAbs) attributes. Graphical representation of the various NAb functions (outside green circle), epitope recognition (inside yellow circle), isotype (inside red circle), and cells shown to produce NAbs (inside blue circle).

Illustration from Nichol A. Holodick, Nely Rodriguez-Zhurbenko, and Ana Maria Hernández. "Defining Natural Antibodies." *Frontiers in Immunology* 8 (July, 2017) art. 872. doi: 10.3389/fimmu.2017.00872.Material is from page 2 and is made available by Creative Commons Attribution (CC BY) License (https:// creativecommons.org/licenses/by/4.0/ legalcode). Changes have been made to font type in the illustration's explanatory text.

Natural antibodies (NAbs),[983] most simply, are those that are found in an organism that are without any known antigenic experience. In other words, they do not have in their cell history—as do those in the adaptive immune system—exposure to an antigen. NAbs are a diverse ('heterogeneous') group but one capable of recognizing as possible dangers both 'self' (i.e., endogenous) and 'non-self' (i.e., exogenous) antigens. A good number of them are called *natural*

[983] Like 'neutralizing antibody' (discussed later), the 'natural antibody' is also abbreviated NAb.

autoantibodies (NAABs), meaning they recognize and react to 'self' molecules as antigens. As to antibody type, most natural antibodies are IgM or IgG (IgG1, IgG2, IgG4, and especially IgG3), but also some IgA (IgA1 and IgA2).[984] Natural IgA has been indicated as perhaps especially important in the immediate response to infection and providing protection against it.[985] NAbs are produced by B-1 cells—both B-1a ($CD5^+$) and B-1b ($CD5^-CD45RA^{lo}$) subtypes. Those NAbs that can recognize glycan epitopes (i.e., the antigen part an antibody attaches to) are common.[986]

Natural antibodies in the body's fight against SARS-CoV-2 have not gotten the same amount of attention as the immune antibodies of the adaptive immune system response. However, NAbs are known to be important early in infection—precisely because they are not antigen-specific. They possess the broad reactivity and variable affinity that allows an immediate response. Because children have a superior ability to rapidly reproduce NAbs it has been suggested that this reality might be important to understanding why they typically fare much better against SARS-CoV-2.[987]

Natural IgM

Natural IgM—what we might term the base NAb—commonly occurs in healthy people and has been the most studied and reported upon.[988] It is produced in neonates, where it immediately can recognize not only a broad range of diverse exogenous pathogens but also autoantigens, including A/B blood group antigens such as discussed below.[989] It is critically important in providing an early immune response by fighting infection using phagocytosis (ingestion of the pathogen), as well as other processes including activation of complement cascade (discussed early and again below).[990] Natural IgM is known as effective serving as antigen-presenting cells (APCs) for priming $CD4^+$ T-cells for Th1 immune responses as the adaptive immune response develops.[991]

But natural IgM levels, which reach adult levels between ages 5–10, decline over time, and may be a factor in why severe COVID-19 cases become more common after the early 40s.[992] However, there appear to be some people in whom the expected changes do not occur even past age 25 (and, indeed, the level of natural IgG in their bodies may rise).[993] In addition to age-related differ-

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[984] For an overview, see Reyneveld, Savelkoul, and Parmentier. "Current Understanding of Natural Antibodies." Also see Palma et al., "Natural Antibodies—Facts Known and Unknown."
[985] Quinti et al., "IgA Antibodies and IgA Deficiency in SARS-CoV-2 Infection."
[986] Holodick, Rodriguez-Zhurbenko, and Hernández, "Defining Natural Antibodies," 2.
[987] Carsetti et al., "The Immune System of Children," 415–16.
[988] Palma et al., "Natural Antibodies—Facts Known and Unknown," 468.
[989] Matricardi, Dal Negro, and Nisini, "The First, Holistic Immunological Model," 460.
[990] Palma et al., "Natural Antibodies—Facts Known and Unknown," 469.
[991] Shivarov, Petrov, and Pashov, "Potential SARS-CoV-2 Preimmune IgM Epitopes," 4.
[992] Matricardi, Dal Negro, and Nisini, "The First, Holistic Immunological Model," 460.
[993] Palma et al., "Natural Antibodies—Facts Known and Unknown," 467.

ences, there seems to be sex-based difference, with males having lower natural IgM levels—and higher rates of severe COVID-19.[994]

Lower IgM levels are also found in individuals belonging to blood group A. That observation returns us to a matter discussed in the chapter on COVID-19—the difference that an individual's blood type can make. Let's look at a quick overview, followed by repeating some of what was reported earlier:

	Blood Type A	**Blood Type B**	**Blood Type AB**	**Blood Type O**
COVID-19 risk	higher	-	-	lower
blood type-related antibodies	anti-B	anti-A	none	anti-A anti-B anti-AB
ABO System Model ***(viral particle profile)***	can infect A can infect AB	can infect B can infect AB	can infect AB	can infect A can infect B can infect AB can infect O

SARS-CoV-2, like other viruses, depends on the host cell for glycan synthesis and modification. In short, for glycosylation to happen in the virus-host interaction, the virus needs the host cell to provide the glycan material that viral glycoproteins—vital to attachment and fusion—require. But this means that replicated virus particles take with them the glycan the host cell supplied—and this is the point where different blood types matter. For example, people with type O blood lack A or B antigens, so when a virus shows up with a glycan-motif derived from these other blood types, type O blood cells treat the newcomer as hostile. Its receptors refuse to recognize the unfamiliar glycan motif of the virus and thereby frustrate attachment and fusion. The host immune system also forms anti-A or anti-B antibodies.[995]

This situation establishes a system of interrelationships among blood groups such that a particular person may be more or less vulnerable to infection by SARS-CoV-2 depending on the blood type of the individual transmitting the virus. As has been pointed out, this is a situation resembling possible 'matched' or 'mismatched' blood transfusions. Inhibition of viral infection may not be 100%, but it does appear to offer some resistance. But once an infection does occur, the newly infected person will produce SARS-CoV-2 viruses of the same ABO phenotype as him- or herself (i.e., the virus profile will look like the blood type of the infected person in which it was produced). This means natural anti-

[994] Matricardi, Dal Negro, and Nisini, "The First, Holistic Immunological Model," 460.
[995] Miotto et al., "Does Blood Type Affect the COVID-19 Pattern?" 15.

bodies may only be relevant against an initial attack and not for subsequent productive infection.[996]

As the opening illustration shows, NAbs are important in many more ways than our brief consideration of them can explore. Among other matters related to COVID-19, NAbs appear to be important in the response to infection in the upper respiratory airways early in infection; individuals deficient in natural IgM and IgA may be more vulnerable to the rapid spread of SARS-CoV-2 and the development of pneumonia.[997] NAbs also have been implicated in the peripheral nervous system manifestations seen in some COVID-19 patients.[998] The long-term duration of the humoral immune response has been reported in some studies as up to 8–12 months after infection for IgG. Although it remains unclear what protection this may confer, it offers the hope of some value against reinfection.[999]

Pattern Recognition Molecules (PRMs)

Earlier we examined a number of PRRs, but we must pause here to consider *pattern recognition molecules* (PRMs) of humoral immunity. These PRMs are 'ante-antibodies'—functional ancestors of antibodies. They recognize and eliminate danger using common mechanisms such as [1] clumping material to surface antigenic components on pathogens (*agglutination*)—an efficient way to bind to multiple pathogens at once; [2] blocking infection by binding to pathogenic attachment sites (*neutralization*); [3] activation of *complement cascade* (discussed elsewhere); and [4] painting a target on a pathogen to attract cells to devour it (*opsonization* facilitating phagocytosis).[1000]

Humoral PRMs include:

- *collectins*—most notably *mannose-binding lectin* (MBL)—are more formally known as *collagen-containing C-type* (calcium-dependent) *lectins*, and constitute an extremely versatile group of nine known PRMs capable of recognizing a diverse range of pathogens;[1001]
- *ficolins* (*M-ficolin* (ficolin-1), *L-ficolin* (ficolin-2), and *H-ficolin* (ficolin-3))—these PRMs recognize carbohydrate molecules on pathogens, apoptotic and necrotic cells;[1002] and,
- *pentraxins*—multimeric proteins grouped as either 'long' or 'short' pentraxins.[1003]

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[996] Yamamoto, Yamamoto, and Muñiz-Diaz, "Blood Group ABO Polymorphism," 2. Also on this matter, and with particular reference to the higher risk for blood group A and lower risk for blood group O, see Shokri et al., "The Relationship Between Blood Groups."
[997] Matricardi, Dal Negro, and Nisini, "The First, Holistic Immunological Model," 459.
[998] Fotuhi et al., "Neurobiology of COVID-19," 13.
[999] Petersen et al., "SARS-CoV-2 Natural Antibody Response," 1–2.
[1000] Stravalaci et al., "Recognition and Inhibition of SARS-CoV-2," 275.
[1001] On collectins, see Murugaiah,. Tsolaki, and Kishore, "Collectins."
[1002] On ficolins, see Zhang and Ali, "Ficolins: Structure, Function and Associated Diseases."
[1003] On pentraxins, see Mantovani et al., "Pentraxins in Innate Immunity."

Collectins

Collectins and ficolins are inferred from available data on their interactions with other coronaviruses to be engaged also against SARS-CoV-2. In such engagement collectins and ficolins are thought to work as PRRs for activating the lectin pathway to complement activity.[1004] Collectin human pulmonary *surfactant protein D* (SP-D), known as a potent viral inhibitor, was found to be present in high levels in serum of the related coronavirus SARS-CoV. For both SARS and coronavirus HCov-229E, SP-D appears to inhibit infection through interacting with the receptor-binding domain (RBD) of the spike (S) protein. It seems reasonable, then, to infer its action against SARS-CoV-2, and a study using *recombinant fragment of human SP-D* (rfhSP-D)—i.e., a small portion of SP-D—found it interacted with the RBD of S protein, and the human host ACE2 receptor the virus prefers to attach to, thus inhibiting infection and replication.[1005]

The collectin *mannose-binding lectin* (MBL)—or more particularly, *serum mannose-binding lectin* (sMBL)—has been found able to interact with SARS-CoV-2 spike (S) protein in such a way as to interfere with viral infection, and also activates the complement lectin pathway.[1006] Human sMBL fights pathogens both directly (by inhibition and by neutralization), and indirectly (by acting as an opsonin painting a target cell for destruction, and by participating in complement activation via the *mannose-binding lectin pathway*). It has been proposed that individuals with genetically determined low levels of sMBL may be more predisposed to successful infection by SARS-CoV-2.[1007] This inference follows from the observation that individuals with *mannose-binding lectin deficiency* are susceptible to severe respiratory infections. One study found exactly this situation in such an individual who was twice infected with SARS-CoV-2.[1008]

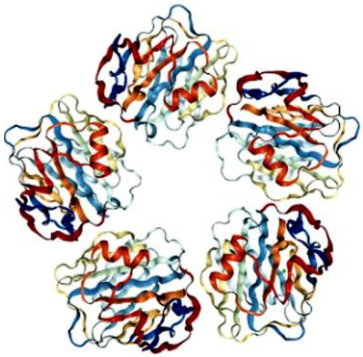
Ficolins

Ficolins (FCNs) are humoral molecules that recognize carbohydrate molecules on pathogens and damaged cells. Higher expression of ficolin-1 (FCN-1) has been found in *bronchoalveolar lavage fluids* (BALFs) in COVID-19 patients.[1009] Ficolin-1, ficolin-2, and ficolin-3 are among the components of the lectin pathway of complement (discussed later). Ficolin-2 (FCN-2) has been demonstrated able to bind SARS-CoV-2 spike (S) and nucleocapsid (N) proteins.[1010] Along with MBL, Ficolin-3 (FCN-3) has been found at higher levels in COVID-19 patients, presumably in association with activation of the complement system.[1011]

[1004] Polycarpou et al., "Rationale for Targeting Complement in COVID-19."
[1005] Madan et al., "A Recombinant Fragment of Human Surfactant Protein D."
[1006] Stravalaci et al., "Recognition and Inhibition of SARS-CoV-2," 276, 281, and Gupta and Gupta, "Status of Mannose-Binding Lectin (MBL)," 2922.
[1007] Gupta and Gupta, "Status of Mannose-Binding Lectin (MBL)," 2923, 2929, and 2936.
[1008] Hayes, Stanley, and Peppers, "COVID-19 Recurrence without Seroconversion."
[1009] Ricci et al., "Innate Immune Response to SARS-CoV-2 Infection," 11.
[1010] Ali et al., "Lectin Pathway Mediates Complement Activation by SARS-CoV-2 Proteins," 6–7.
[1011] Malaquias et al., "The Role of the Lectin Pathway of the Complement System," 59–60.

Perhaps the best known PRMs that interact with PAMPs belong to an important multimeric protein family—the *pentraxins*. The name, coined from a clever combination of Greek words, reflects the appearance of the distinctive structure—'five' (*penta-*) 'berries' (*-ragos*).[1012]

Illustration extracted from Zeyu Wang, Xing Wang, Hecun Zou, Ziyu Dai, Songshan Feng, Mingyu Zhang, Gelei Xiao, Zhixiong Liu, and Quan Cheng. "The Basic Characteristics of the Pentraxin Family and Their Functions in Tumor Progression." *Frontiers in Immunology* 11 (Aug., 2020) art. 1757. doi: 10.3389/fimmu.2020.01757. The excerpted portion is from p. 2, Figure 1, and reproduces their image of the protein structure of CRP. The material is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/ legalcode).



This family has two subgroups: short pentraxins and long pentraxins (the latter about twice as long in their protein sequences). Various members of the short pentraxins have been implicated in immune system regulation (e.g., *C-reactive protein* (CRP) and *serum amyloid P component* (SAP)). A member of the long pentraxins—*Pentraxin 3* (PTX3)—inhibits cell proliferation in cancer and appears to be a highly sensitive inflammation-related factor.[1013] In fact, the role of pentraxins in inflammation, and the ability of a pentraxin (CRP) to activate the classical cascade of complement (discussed later),[1014] makes this group of keen interest with respect to SARS-CoV-2 and COVID-19. Both CRP and PTX3 have been identified as biomarkers of short-term mortality associated with SARS-CoV-2 infection in intensive care COVID-19 patients.[1015]

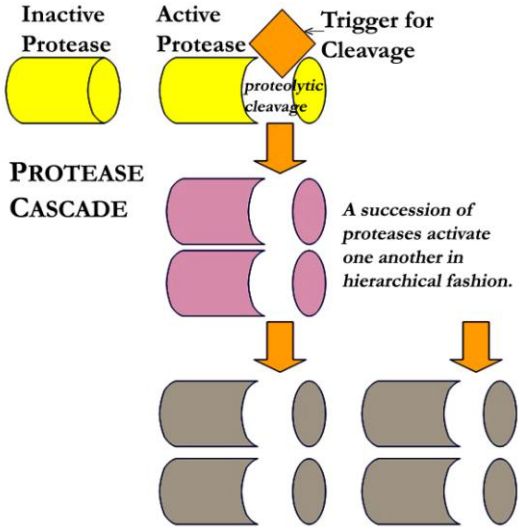
PTX3 is a pattern-recognition protein encoded by the PTX3 gene. PTX3 is produced in a variety of cell types (e.g., myeloid cells, endothelial cells and respiratory epithelial cells), and can be done so rapidly. It has been found expressed at high levels by myeloid cells in blood and lungs in SARS-CoV-2 infection. The virus induces gene expression of PTX3 in epithelial (i.e., surface level) cells of the respiratory tract. It binds some viruses and plays a role regulating inflammation. Although it recognizes the nucleocapsid (N) protein of SARS-CoV-2, it does not seem to exhibit antiviral activity there. But it occupies an important position at the intersection between macrophage-driven inflammation and vas-

[1012] Not '*penta*' + '*axin*'; see Pepys, "The Pentraxins 1975–2018," 2 (footnote 2).
[1013] Wang et al., "The Basic Characteristics of the Pentraxin Family."
[1014] On both the relation to inflammation and complement, see Du Clos, "Pentraxins."
[1015] Schirinzi et al., "Pentraxin 3: Potential Prognostic Role," and Moulana et al., "Increased Levels of Serum Pentraxin 3 in Critical Coronavirus Disease-2019 Patients," 4.

cular involvement. With respect to COVID-19 it has been linked to the Kawasaki-like disease in children now known as *multisystem inflammatory syndrome*. Elevated plasma PTX3 levels have been associated with a severity of pathology in Kawasaki disease and in COVID-19, with prognostic significance for mortality in the latter.[1016]

Serine Protease Cascades, 1: Complement

Serine is an α–amino acid important in the bio-synthesis of proteins. *Serine proteases* are enzymes (a kind of protein) that sever ('cleave') protein peptide bonds. They are very common; almost a third of proteases (catalyzing enzymes) are serine proteases.[1017] A *protease cascade* (pictured at left) occurs when a succession of proteases activate one another in hierarchical fashion via the process of proteolytic cleavage.



Our interest here lies in two kinds of serine protease cascades:

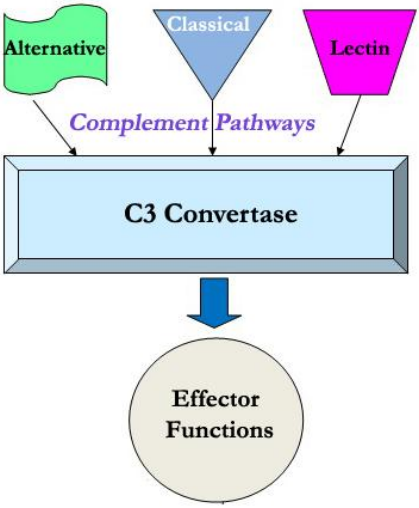
- complement, and
- contact system.

Complement, also referred to as *complement system*, we have mentioned numerous times. Complement is comprised of a large group (perhaps more than four dozen) of soluble and cell-surface proteins[1018] that enhance—'complement'—other immune actions (e.g., by antibodies). Complement can be activated either directly by pathogens or indirectly by pathogen-bound antibodies.

It can be said to have three broad functions:

- activation of inflammation;
- targeting (*opsonization*) of pathogens and damaged cells for removal; and,
- direct destruction of pathogens or target cells by rupturing their protective membrane (*lysis*).[1019]

[1016] Brunetta et al., "Macrophage Expression and Prognostic Significance of the Long Pentraxin PTX3 in COVID-19," and Stravalaci et al., "Recognition and Inhibition of SARS-CoV-2," 281.
[1017] For an overview, see Hedstrom, "Serine Protease Mechanism and Specificity."
[1018] Ricklin et al., "Complement Component 3" puts the number at about 50; cf. Sim and Tsiftsoglou, "Proteases of the Complement System," or the more recent Bardhan and Kaushik, "Physiology, Complement Cascade."
[1019] Monie, *The Innate Immune System*, 23.



Complement operates in plasma, in tissues, or within cells.[1020] Three activating pathways have been identified, which can be symbolically illustrated in broad strokes (at right).[1021]

The *classical pathway* (CP) derives its name from the historical reality that it was the first pathway described and for some time was believed to be the only complement pathway. It is activated by an *immune complex* (the binding of multiple antigens to antibodies, hence also termed *antigen-antibody complex*, starring either IgG or IgM), or by pentraxins.[1022]

The *alternative pathway* (AP), discovered in the early 1950s, earned its name by being found an 'alternative' to CP. In fact, it has been found to be the most ancient of the three pathways and is regarded as logically primary since it is active by default—meaning it effectively activates and amplifies wherever allowed; surfaces able to regulate complement stop its activation.[1023] In plasma, during normal physiological conditions, AP is the dominant complement pathway.[1024]

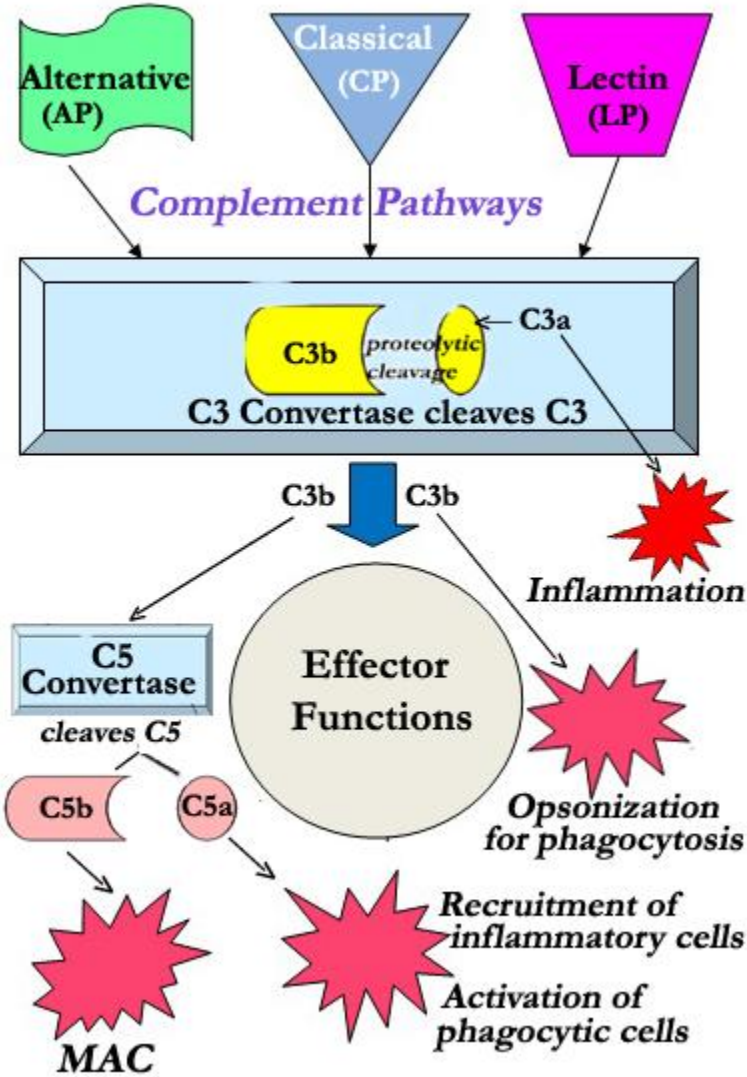
The *lectin pathway* (LP), as the name indicates, is activated by a lectin (carbohydrate-binding protein)—specifically *mannose-binding lectin* (MBL), which we met a little earlier. Other involved participants include ficolins (FCNs) we discussed above.

All three pathways utilize a versatile molecular scaffold—*complement component 3* (C3)—that has been called the 'Swiss Army knife' of innate immunity.[1025] The serine protease *C3 convertase* (C4bC2b) is an enzyme occupying a central role in complement cascade and is called a 'convertase' because it causes a change to C3 ('converts' it). Through cleavage the C3 convertase first changes C3, which is inactive, into two active components of unequal sizes: C3a and C3b. The larger 3b is able to bind to the surface of pathogens or damaged cells. Next, the convertase can bind C5 and function as a *C5 convertase*. Each pathway contains a C3 convertase that first cleaves C3, producing C3b and leading to a

[1020] Merle et al., "Complement System Part I," 1.
[1021] The illustration is a reduced and simplified one based on Monie, *The Innate Immune System*, 24.
[1022] Mosquera-Daza, "A Look at the C3 and C5 Proteins in the COVID-19," 3.
[1023] Ferreira and Cortes, "The Complement System," 144–45.
[1024] Merle et al., "Complement System Part I," 2.
[1025] Ricklin et al., Complement Component C3."

C5 convertase. Thus these pathways engage two proteins (C3 and C5) that mediate almost all complement effector functions.[1026]

With these things in mind we can modify our previous (simplified) illustration to look like the following:



[1026] Zwarthoff et al., "Functional Characterization of Alternative and Classical Pathway," 2. Also see Mosquera-Daza, "A Look at the C3 and C5 Proteins in the COVID-19," 4–6.

Normally, complement is a feature of ordinary body operations as the pathways are activated on the surface of apoptotic cells—those scheduled to die to maintain health. It is thus essential to normal cellular homeostasis. But when pathogens invade complement is more fully activated and leads to the functions listed earlier: inflammation, opsonization, phagocytosis, and destruction of the pathogen also associated with activation of the adaptive immune system.[1027]

Let us venture a little deeper with a bit more detailed illustration to highlight some of the ideas being discussed.

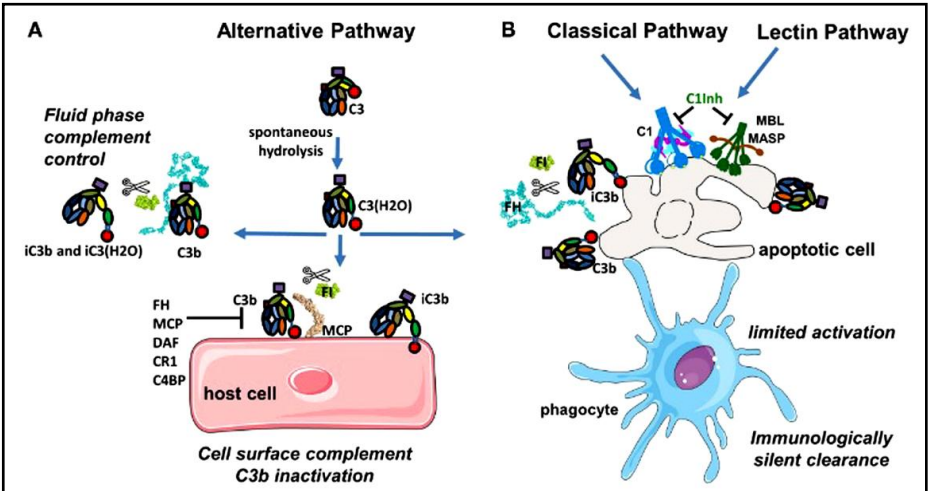


Figure 1 | Complement activation in physiological conditions. **(A)** The alternative pathway is permanently active due to spontaneous transformation of bio-inactive molecule C3 to bioactive C3(H_2O). This allows generation of C3b, which is rapidly inactivated by FH and FI in fluid phase or is covalently bound to the surface and then inactivated on host cells. **(B)** Classical and lectin pathway recognition molecules bind to apoptotic cells and together with C3b from the alternative pathway induce a low level of complement activation. Apoptotic cells are not lysed, but rapidly cleared by phagocytes in an immunologically silent manner. Host cells and plasma contain multiple regulatory proteins to assure that complement activation will be limited in physiological conditions.

Illustration from Nicolas S.Merle, Sarah Elizabeth Church, Veronique Fremeaux-Bacchi, and Lubka T. Roumenina. "Complement System Part I—Molecular Mechanisms of Activation and Regulation." *Frontiers in Immunology* 6 (June, 2015) art. 262. doi: 10.3389/fimmu.2015.00262. The figure is from p. 2. The material is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/ 4.0/ legalcode). No changes have been made save for alteration of font style and size.

The powerful mechanisms entailed in complement mean it must be tightly regulated. If not—as can happen with infection—the complement system that

[1027] Merle et al., "Complement System Part I," 1–2.

guards health can become a threat to health. Regulation transpires at multiple levels and relies on various inhibitors such as *factor H* (FH) interacting with *C-reactive protein* (CRP)—a pentraxin mentioned earlier. Once more, C3 figures prominently—or more precisely, its large C3b fragment. When it is cleaved a fragment called *inactivated* C3b (iC3b) results. Like C3b, this iC3b fragment can serve as an opsonin to mark target cells for phagocytosis (destruction through ingestion).[1028] The illustration below elaborates on this process of regulation.

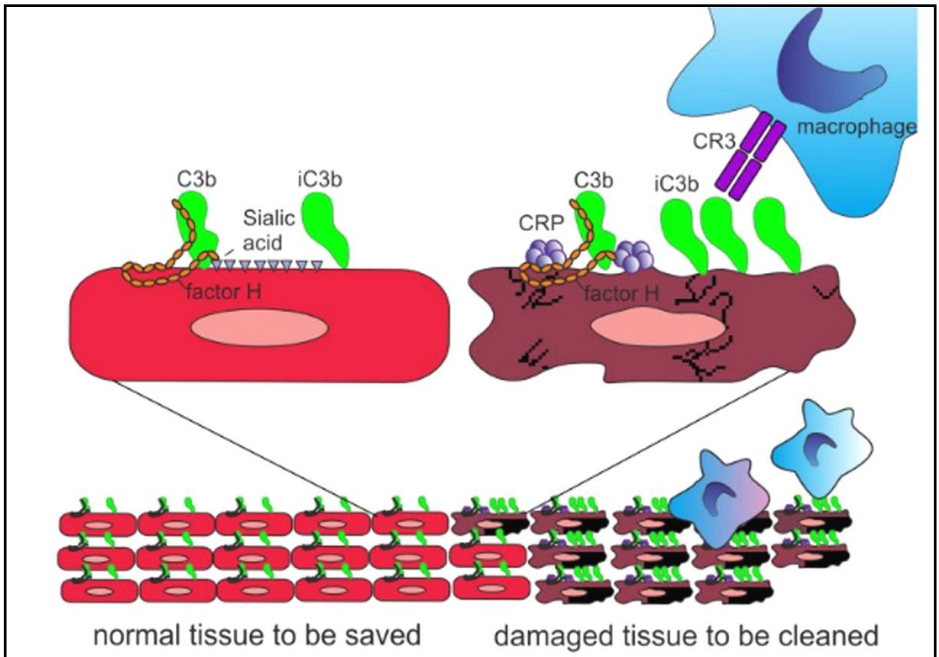


FIGURE 4 | Role of CRP and FH in promoting clearance of dying cells. On viable cells (Left) accidentally deposited C3b is rapidly inactivated to iC3b and no C activation takes place. On surfaces, from where protective polyanions, like sialic acids, are lost as a consequence of cell damage, factor H binding is decreased (Right). This together with CRP-mediated classical pathway activation leads to complement activation. During a prolonged time course, the large number of deposited C3b molecules, will, however, be inactivated to iC3b with the help of factor H recruitment by CRP. Deposited iC3b molecules promote phagocytosis of the debris without leading to further activation of the terminal pathway.

Illustration from Karita Haapasalo and Seppo Meri. "Regulation of the Complement System by Pentraxins." *Frontiers in Immunology*, 10 (2019) art. 1750. DOI:10.3389/fimmu. 2019.01750. The figure is from p. 8. The material is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/ 4.0/ legalcode). No changes have been made save for alteration of font style and size.

[1028] On this matter, see Jensen et al., "Complement Receptor 3 Forms a Compact High-Affinity Complex with iC3b."

As all of the above has explained, *complement cascades* are a kind of proteolytic cascade in which serine proteases are activated by one another. A complement cascade is initiated by recognition of some danger (e.g., as briefly mentioned earlier in looking at *complement-dependent cytotoxicity* (CDC)). Following such recognition, serine proteases are activated. These act in concert to initiate and propagate the cascade with varied effector functions thus engaged.[1029]

Complement can be activated by SARS-CoV-2 both directly and indirectly. Direct activation can happen because both spike (S) and nucleocapsid (N) proteins can be recognized by lectin pathway (LP) components, leading to complement activation. Also, IgG and IgM antibodies directed against the receptor-binding domain (RBD) of the S protein are able to initiate classical pathway (CP) activation. Moreover, S protein can dysregulate the alternative pathway (AP) of complement by interacting with a negative regulator of complement activity (*heparan sulfate*). Indirect activation of complement by SARS-CoV-2 is achieved by causing injury and dysfunction to endothelial cells (*endotheliopathy*) and by inflammation associated with coagulation and blood clots (*thromboinflammation*), which activate complement.[1030]

During early infection by SARS-CoV-2, complement is activated in lung tissue, triggering the formation of a *membrane attack complex* (MAC) able to disrupt the virus' membrane (*lysis*). The presence of C3a and C5a recruits circulating leukocytes to migrate to the site of inflammation. The C3a and C5a fragments also trigger the release of inflammatory mediators by mast cells. C3b-coated viral material binds to *complement receptor* (CR) found in phagocytes, which proceed to ingest the viral material so marked. Generally, these actions control infection. When they do not, as seen in severe COVID-19, the inflammatory response can become exaggerated and lead to cytokine storm and extreme tissue damage.[1031]

Thus, complement is another of the immune system's double-edged swords. Though often a useful weapon against danger, it can also be dangerous to the user. It has been implicated in the pathogenesis of COVID-19 with its overactivation associated with respiratory failure and a serious blood clotting disorder (*intravascular coagulopathy*).[1032] The endothelial injury and hypercoagulability observed is associated with the cross-talk between the complement and coagulation systems, with complement AP especially indicted. Research suggests what is seen in severe COVID-19 in this respect of higher complement activation is unusual and not characteristic of similar diseases such as severe influenza or other forms of acute respiratory failure.[1033]

[1029] Sim and Laich, "Serine Proteases of the Complement System."
[1030] Afali et al., "The State of Complement in COVID-19," 77.
[1031] Santiesteban-Lores et al., "A Double-Edged Sword," 7 (Fig. 3).
[1032] Defendi et al., "Complement Alternative and Mannose-Binding Lectin Pathway," 4.
[1033] Ma et al., "Increased Complement Activation Is a Distinctive Feature," 5–6.

Serine Protease Cascades, 2: Plasma Contact System

The *contact system* (CS) derives its name from becoming active in *contact* with certain surfaces. CS consists of a group of serine proteases (and their substrates) that assemble on the surfaces of circulating blood cells and vessel walls,[1034] or put a bit differently, it consists of plasma proteins found within the four chambers of the heart and the body's vascular system (the *intravascular compartment*). Factors of the CS either bind directly on viral surfaces or on infected cells.[1035]

Contact System Components

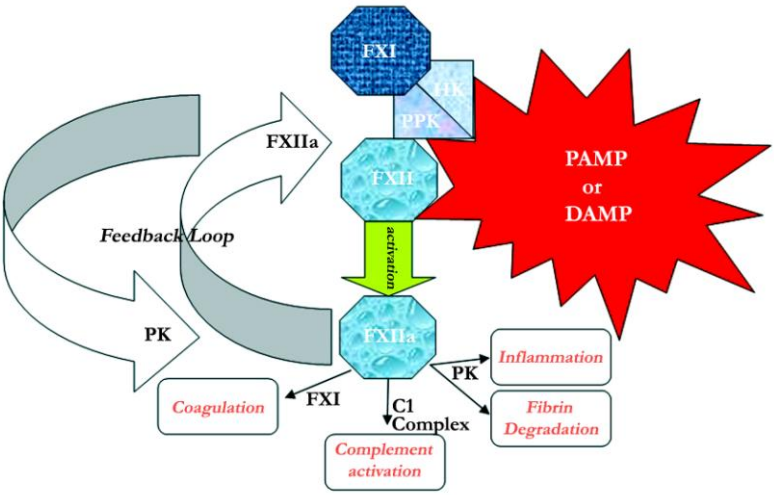
CS's principal players, proteins produced by the liver and then circulating in the bloodstream in an inactive state (as *zymogens*), and a cofactor, are:

- serine protease *factor XI* (FXI when zymogen), which when activated is FXIa;
- serine protease *factor XII* (FXII when zymogen), which when activated is FXIIal;
- serine protease *plasma prekallikrein* (PPK when zymogen), which when activated is *plasma kallikrein* PK); and,
- serine protease *C1 esterase inhibitor* (C1INH) a regulator, plus
- non-enzymatic cofactor *high-molecular-weight kininogen* (HK).

Most PPK—about 75%--circulates in complex with HK.[1036]

Contact System Operation

Let's start with a simple picture (followed by some explanation).



[1034] Weidmann et al., "The Plasma Contact System," 2118.
[1035] Meini et al., "Understanding the Pathophysiology of COVID-19," 2.
[1036] Long et al., "Contact System Revisited," 427; Meini et al., "Understanding the Pathophysiology of COVID-19," 2–3; Wu, "Contact Pathway of Coagulation and Inflammation," 1.

Inactive FXII becomes activated FXIIa by contact with negatively charged surfaces such as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). HK (complexed with PK) binds to the surface of the PAMP or DAMP. The activated FXIIa cleaves inactive PPK to form active PK, which activates more FXII in a feedback loop. The conformational change in activated PK allows it to act as an enzyme and cleave HK, which then releases *bradykinin* (BK), a potent proinflammatory peptide—and the end product of the kallikrein-kinin pathway.[1037] Among the other things bradykinin does, it increases inflammation and complement activation.[1038]

The Contact System and SARS-CoV-2

The contact system can be activated as a result of activation of complement following endothelial cell damage from viral infection. Some viruses are known to modulate the contact cascade to promote infection,[1039] and it is reasonable to think SARS-CoV-2 does so. CS promotes coagulation and inflammation with sufficient activation of FXII,[1040] and the expression of FXIIa increases in lung tissue in COVID-19. Further, FXII, PK and HK, together with a multifunctional protein (gC1qR), have been reported to form an *HK/FXII/gC1qR complex*, which could participate in coagulation or inflammation.[1041] Studies have indicated that severe cases of COVID-19 show marked levels of CS activation leading to hypercoagulation (a pronounced tendency toward blood clotting blocking veins or arteries (*thrombosis*)).[1042]

The *C1 esterase inhibitor* (C1INH), the other major player named earlier, is the major regulator of the CS (and also regulates the classical pathway of the complement system).[1043] Studies have shown that C1INH is an interactor for seven distinct SARS-CoV-1 proteins and polypeptides, and that these proteins are highly similar to those of SARS-CoV-2. These interactions may mean inhibition of C1INH during infection, with SARS-CoV-2 possibly causing deficiency in C1INH—all of which increases the likelihood of the contact system producing bradykinin and activating both the complement system and the intrinsic coagulation cascade.[1044]

The contact system is not nearly as well known as complement, nor has it received the same degree of attention. With respect to SARS-CoV-2 and COVID-19 it continues to have limited coverage in scientific literature.[1045]

[1037] Long et al., "Contact System Revisited," 427; Meini et al., "Understanding the Pathophysiology of COVID-19," 2–3; Wu, "Contact Pathway of Coagulation and Inflammation," 2.
[1038] Mackman et al., "Coagulation Abnormalities and Thrombosis in Patients Affected with SARS-CoV-2," 2038–39.
[1039] Shishido et al., "Humoral Innate Immune Response and Disease," 147.
[1040] Meini et al., "Understanding the Pathophysiology of COVID-19," 3.
[1041] Hernández-Huerta et al., "Mechanisms of Immunothrombosis by SARS-CoV2," 3.
[1042] Busch et al., "Neutrophils and Contact Activation of Coagulation."
[1043] Schoenfeld, Lahrsen, and Alban, "Regulation of Complement and Contact System."
[1044] Thomson et al., "C1 Esterase Inhibitor and the Contact System in COVID-19."
[1045] Maglakelidze, Manto, and Craig, "A Review," 173.

Summing Up the Innate Immune System Response

One final illustration for this section, focused on innate immune system response in the part of the body SARS-CoV-2 most famously troubles—the lungs—may be useful for drawing together various parts of what we have examined. (The illustration's explanation continues on the next page.)

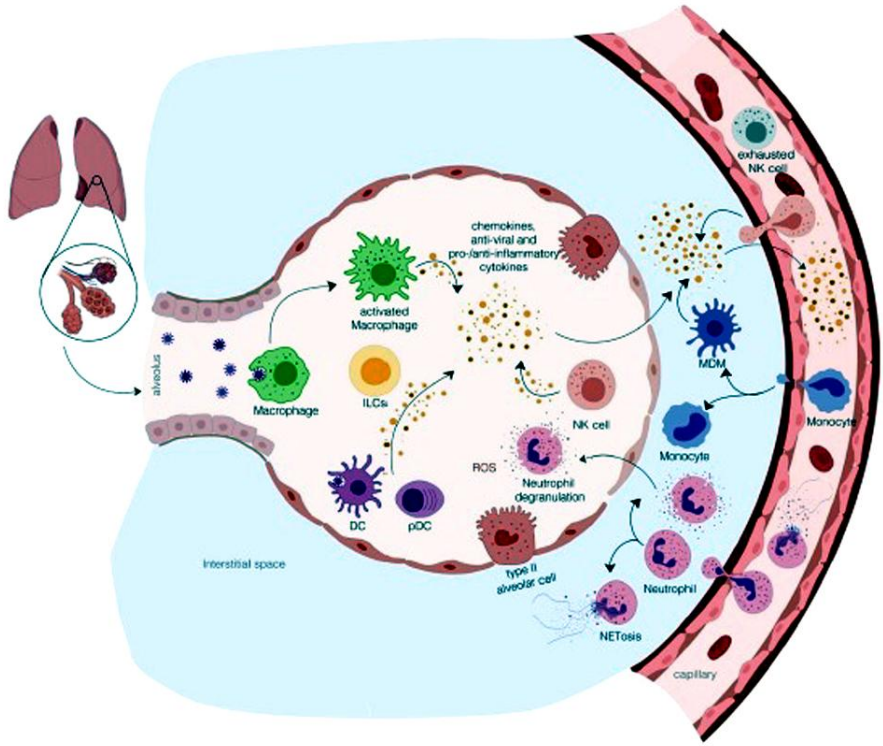


Figure 4. Innate immune response in SARS-CoV-2-infected lung. Aerosolized uptake of SARS-CoV-2 allows the infection of angiotensin-converting enzyme 2 (ACE2)-expressing target cells in the lung such as alveolar type II cells. A successful control of viral spread relies on the induction of local IFN-dependent antiviral state and the activity of alveolar macrophages that neutralize viruses and infected apoptotic cells and clear them by phagocytosis. The initial inflammation induced by the release of soluble factors, including proinflammatory cytokines and chemokines, from resident immune cells and infected epithelial cells attracts and activates neutrophils, monocytes, macrophages, dendritic cells (DC), natural killers (NK), and innate lymphoid cells into the site of infection, where they contribute to the elimination of the infected cells before virus spreading. If the virus takes over by dampening the IFN antiviral effect, the proinflammatory response increases because of the further infiltration of monocytes/macrophages, neutrophils, and several other adaptive immune cells from the bloodstream, resulting in "cytokine storms". In addition, the formation of neutrophil extracellular traps (NETs)—aggregates composed by extracellular DNA fibers, histones,

microbicidal proteins, and proteases released from the recruited neutrophils—can further trigger and sustain the local inflammation. Thus, the early phase of SARS-CoV-2 interaction with host cells, including innate immune cells, is crucial in determining the fate of the infection spanning from minimal lung damage with recovery and a maintaining of vascular integrity to the disruption of lung structure associated with pulmonary edema and pneumonia.

Illustration from Daniela Ricci, Marilena Paola Etna, Fabiana Rizzo, Silvia Sandini, Martina Severa and Eliana Marina Coccia. "Innate Immune Response to SARS-CoV-2 Infection: From Cells to Soluble Mediators." *International Journal of Molecular Sciences* 22 (2021) 7017. https://doi.org/10.3390/ ijms22137017. Material is from page 10 and is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/ legalcode). Changes have been made to font type in the illustration's explanatory text.

Why Isn't the Innate Immune Response Enough?

Researcher Christopher Coleman points out, "One of the key interactions in the pathogenesis of any viral infection is that between the virus and the host innate immune response. An effective innate immune response can clear the virus before infection is established. Therefore, most viruses have evolved mechanisms to block and/or antagonize the innate immune response."[1046] Researchers Alessandro Sette and Shane Crotty add that without some trick to evade the immune system a virus is harmless, but SARS-CoV-2 clearly is not. They remark that its 'big trick' is that it "is clearly unusually effective at evading the triggering of early innate immune responses."[1047]

As we have seen, the virus messes with the production of the very interferons that might 'interfere' with its replication. The resulting dysregulation sometimes gets so out of hand as to result in catastrophic cytokine storms. Yet the bag of tricks runs deeper than a single bit of sleight of hand. SARS-CoV-2 may even hijack the very defense actions the body intends against it and turn those acts to its own advantage, as we saw in how it interacts with dendritic cells. Such viral mechanisms of exploiting vulnerabilities in the innate immune system remind us why just that immune response may not prove sufficient. Plus, RNA viruses like SARS-CoV-2 mutate rapidly and continue to do so as they interact with their hosts. Thus, the potential for increasingly effective means of evading, blocking, or co-opting immune defenses remains.

Fortunately, the innate immune system works synergistically with the adaptive immune system. For example, when SARS-CoV-2 establishes its beachhead in the human respiratory system tissue-resident cells of the innate immune system at once offer resistance in such a manner as to recruit other innate immune cells from the blood.[1048] But they also interact with adaptive immune cells.

[1046] Coleman, "Determining How Coronaviruses Overcome the Interferon and Innate Immune Response," 223.
[1047] Sette and Crotty, "Adaptive Immunity to SARS-CoV-2 and COVID-19," 861.
[1048] Ricci et al., "Innate Immune Response to SARS-CoV-2 Infection," 3.

The adaptive immune system is primed by the innate immune system response to SARS-CoV-2. The most well-known stars of the adaptive immune system are antibody-producing B-cells. Antibodies—scientifically referred to as *immunoglobulins*—play a starring role in the fight against SARS-CoV-2. Most, but not all, of those who are infected by SARS-CoV-2 develop antibodies against it that later are evident in their blood.

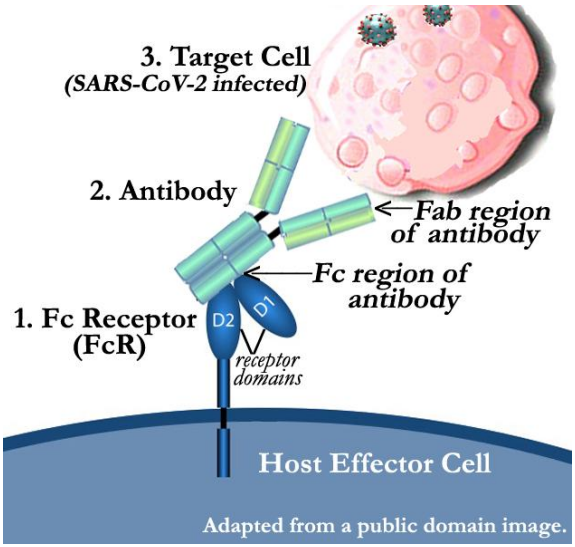
Let us start by re-examining the mechanism and process by which antibodies accomplish their work—*binding* to a pathogen.

Antibody (Ab) Binding and SARS-CoV-2

Antibodies, remember, have two specific regions dedicated to special tasks:

- The *fragment antigen-binding* (Fab) domain contains the place (the *variable region*) where binding of the B-cell to its antigen occurs; an antibody has two such regions.
- The *fragment crystallizable* (Fc) *region* is a region (the *constant region*) that binds to various cell receptors as well as to certain proteins of the *complement system* (or simply *complement*) in order to mediate effector functions of other immune system parts dedicated to removing perceived danger.

Against SARS-CoV-2, it is not an 'either-or' proposition but a 'both-and' as antibodies employ all their parts and functions. Let's revisit an earlier illustration, now slightly altered, to accompany an explanation.



The Fab region, recall, is responsible for ensuring that a suitable response occurs to the viral agent (the *antigen*) provoking the host immune system. This region of the antibody binds to the antigen—in SARS-CoV-2 the spike (S) protein (or some portion of it).

Meanwhile, the Fc region of the antibody is linking both to an infected target cell and to a host effector cell's *Fc receptors* (FcRs). In this manner an immune response can be activated that stimulates 'devouring cells' (*phagocytes*) and 'poisonous to other cells' (*cytotoxic*) cells to accomplish their functions against SARS-CoV-2.

B-Cell Produced Non-Neutralizing Antibodies (n-NAbs)

Antibodies 'bind' to things, but as we have seen above not all the 'binding' is the same. In general it is convenient to distinguish antibody binding this way:

- *Binding antibodies* (or, *non-neutralizing antibodies* (n-NAb)) work (unsuccessfully) to prevent infection by SARS-CoV-2 entering host cells, but serve as biomarkers of infection taking place.
- *Neutralizing antibodies* (NAb)[1049] live up to their name by 'neutralizing'—rendering impotent—SARS-CoV-2.

Most of the antibodies elicited by an infection are non-neutralizing.[1050]

Binding antibodies fail to neutralize because they bind to targets on the virus that are not actually key to viral entry. Since they don't bind to the essential viral parts (like the spike (S) protein) they can't prevent infection from occurring. But that hardly means they are useless. *Non-neutralizing antibodies* (n-NAbs) mediated by Fc-receptor binding to immune cells contribute significantly to antiviral activities through various mechanisms of action.

Let's revisit the matter of how the antibody's Fc region works with immune system effector cells to accomplish actions against the infected cells the antibody is also bound to. In the last chapter we briefly discussed a couple of such actions, which we shall now revisit starting with a simple Table overview.

Fc-FcR interaction results in. . .	**which means. . .**
antibody-dependent cellular cytotoxicity (ADCC)	target cell (TC) poisoning (*cytotoxicity*) by an immune cell resulting in the TC's death.
antibody-dependent cellular phagocytosis (ADCP)	TCs are tagged by antibodies (*opsonization*) to induce phagocytes to ingest (*phagocytize*) the TCs, resulting in their death.
antibody-dependent cellular trogocytosis (ADCT)	TCs become 'donor' cells and are 'nibbled' (*trogocytosis*) to death (*trogoptosis*) by their 'recipients.'
Fc-Complement interaction results in . . .	**which means . . .**
antibody-dependent complement deposition (ADCD)	a membrane attack complex (MAC) is created to destroy a TC.

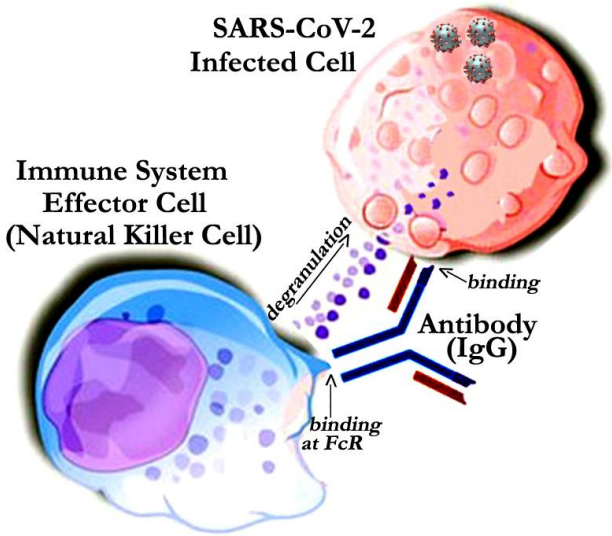
Note that the 'AD-' portion of each abbreviation—'antibody-dependent'—reflects the key and common element: they all rely on the Fc region of the antibody.

[1049] Like 'natural antibody' the 'neutralizing antibody' is also abbreviated NAb.
[1050] Bachmann et al., "SARS-CoV-2 Structural Features May Explain."

In each of the above interactions a two-step mechanism operates: the antibody binds to a target cell (TC), and the Fc domain of the antibody binds to FcγR on the surface of an immune cell (or to complement factors).

Fc-FcR—Antibody-Dependent Cellular Cytotoxicity (ADCC)

Antibody-dependent cellular cytotoxicity (ADCC) we briefly examined in the last chapter. The Fc-FcR (specifically, FcγR) interaction involves an innate immune system effector cell (e.g., macrophages, neutrophils, or natural killer cells), while the antibody is also binding to viral protein on the surface of an infected cell. In the illustration, a natural killer cell (NK), which seems to be the most common immune effector cell employed in ADCC, is induced to release cytotoxic ('poisonous to cells') granules containing perforin and granzymes to kill a SARS-CoV-2 infected target cell (TC).

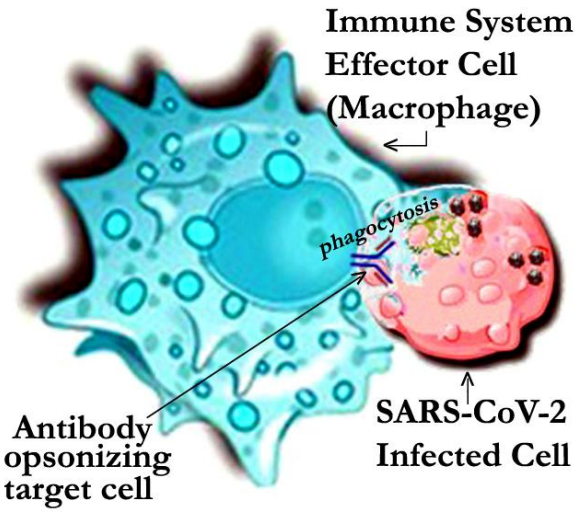


ADCC presumably occurs against SARS-CoV-2 when specific glycoprotein targeting antibodies (IgG n-NAbs), via natural killer (NK) cells, induce it. This has been observed *in vitro* ('in glass'—i.e., outside the body, as in laboratory tests), but remains not strongly confirmed *in vivo* (in the body).[1051] One study demonstrated that ADCC antibody response can occur by 7 days after symptom onset and continues to increase until the early convalescent phase.[1052] In another study, of 255 COVID-19 patients, ADCC was found to be elicited by 10 days after infection, peaked by days 11–20, stayed stable 6–12 months, and remained detectable up to 462 days (some 15 months) after infection.

ADCC, alongside neutralizing antibody action contributes to enhanced control and clearance of SARS-CoV-2. But while neutralizing antibodies can be high, if ADCC is deficient severe COVID-19 may result.[1053] It may be that such an elevated response contributes to pathology by inducing inflammation. Possibly such elevated ADCC is the result of high viral loads that result in prolonged antigen exposure for patients. The whole matter remains under scrutiny.[1054]

[1051] Tso et al., "Presence of Antibody-Dependent Cellular Cytotoxicity (ADCC)."
[1052] Chen et al., "The Development and Kinetics of . . . (ADCC)," 7.
[1053] Vigón et al., Impaired Antibody-Dependent Cellular Cytoxicity," 7–8.
[1054] Yu et al., "Antibody-Dependent Cellular Toxicity Response to SARS-CoV-2."

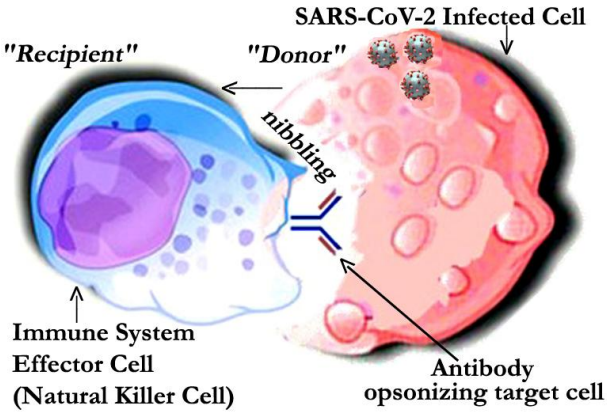
Antibody-dependent cellular phagocytosis (ADCP) was something else we touched on previously. The immune cells with FcRs are macrophages or other phagocytic cells. These are activated by the process of *opsonization* to destroy the target cell (TC) through phagocytosis. Put a little differently, this means the antibody (e.g., IgG) acts as an *opsonin*—a marker serving as an activating agent that induces a phagocytic cell to engulf and 'eat' (*phagocytosis*) the TC. Because the antibody is binding both to the TC and to an effector immune cell (the phagocyte) it is able to bridge them in a manner conducive to the four stages of phagocytosis: 'tasting,' 'feeling,' 'swallowing,' and 'digesting.'[1055]



ADCP has been measured in the serum of COVID-19 patients and found to show higher levels in adults compared to pediatric patients.[1056] Some research has found that ADCP (and ADCC) are most strongly correlated with FcγR-binding antibodies, IgG1, and IgG3.[1057] Research also indicates that monocyte ADCP remains important in contributing to clearing SARS-CoV-2 infection and offering a measure of protection for those who have been vaccinated—and that this remains so across COVID variants.[1058]

It might be well to note here that *antibody-dependent neutrophil phagocytosis* (ADNP) also has been studied with relation to COVID-19. It features antibody Fc interaction with FcγR3b on neutrophils. It has been found at increased levels in patients who died from COVID-19.[1059] Another study found that depletion of IgA reduced ADNP significantly among those with severe COVID-19. Depletion of IgG was found to do similarly with ADNP in both adults and children with mild COVID-19. But when depletion of IgG is combined with the same for IgA it results in complete loss of ADNP in adults with severe COVID-19.[1060]

[1055] Underhill and Goodridge, "Information Processing During Phagocytosis."
[1056] Pierce et al., "Immune Responses to SARS-CoV-2 Infection," 1, 4.
[1057] Natarajan et al., "SARS-CoV-2 Antibody Signatures," 8.
[1058] Kaplonek et al., "mRNA-1273 Vaccine-Induced Antibodies," 356.
[1059] Atyeo et al., "Distinct Early Serological Signatures," 525.
[1060] Bartsch et al."Humoral Signatures of Protective and Pathological SARS-CoV-2," 456–57.



Antibody-dependent cellular trogocytosis (ADCT) can lead to 'death by nibbling' (*trogoptosis*) as the 'donor' cell has surface material taken by the 'recipient' cell—the process of 'cell-nibbling' (*trogocytosis*) that need not always be fatal.

The general process of trogocytosis presents us with a dual identity as both 'friend' and 'foe.' In the former role, it is part of the immune response to pathogens like SARS-CoV-2. On the other hand, it can turn to the role of foe as pathogens use it to escape immune surveillance.[1061]

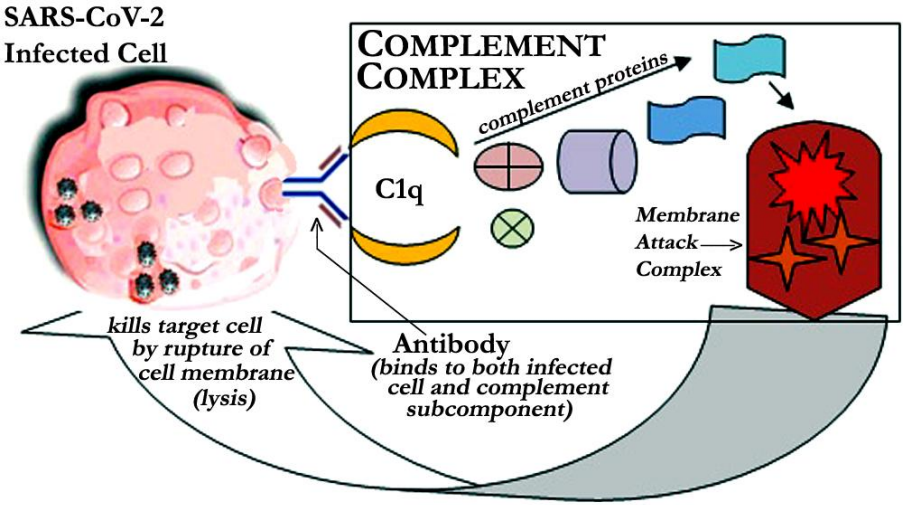
There are a variety of different forms and functions associated with cellular trogocytosis, including antigen presentation, T-cell differentiation, immune regulation, and anti-infection and anti-tumor immunity.[1062] Some of these are especially pertinent to our present concern with 'antibody-dependent' trogocytosis, where FcγR-expressing immune cells (neutrophils, monocytes, macrophages, and NK cells) act as recipients of target cell (TC) donors, with antibodies acting as opsonizing agents.[1063] It is thought that ADCT can enhance antigen presentation, a feature that facilitates immune response,[1064] as the 'nibbled' portion of TC can then be expressed on the surface of the effector cell. This process of nibbling and transfer of material from the TC can disrupt its membrane to the point of causing TC's death. It has been shown that the spike (S) protein of SARS-CoV-2 can induce trogocytosis.[1065]

To date the role of ADCT against SARS-CoV-2 remains largely unknown, but since trogocytosis can result in the transfer of surface cell molecules (cell membrane fragments) to the recipient cell rather than cell death, *trogocytosis-mediated material transfer* is a function also being looked at closely.[1066] Some research shows that, like many other things, ADCT response varies according to interaction of the body with different variants of SARS-CoV-2.[1067]

[1061] Zhao et al., "Gnawing Between Cells," 5.
[1062] Zhao et al., "Gnawing Between Cells," 1.
[1063] On the whole subject, see Miyake and Karasuyama, "The Role of Trogocytosis."
[1064] Richardson and Moore, "Targeting Fc Effector Function in Vaccine Design."
[1065] Gunn and Bai, "Building a Better Antibody through the Fc," 4330.
[1066] On the difference between trogocytosis outcomes, see Zohar and Alter, "Dissecting Antibody-Mediated Protection Against SARS-CoV-2," 393 (Fig. 1). Also see Reed, Reichelt, and Wetzel, "Lymphocytes and Trogocytosis-Mediated Signaling."
[1067] Richardson et al., "SARS-CoV-2 Beta and Delta Variants Trigger Fc Effector Function," 3–4.

Fc-Complement—Antibody-Dependent Complement Deposition (ADCD)

Finally, *antibody-dependent complement deposition* (ADCD), is another result we looked at last chapter. The antibody, already bound to a target cell, uses its Fc region to bind to a subcomponent (C1q) of the C1 complement complex. Then recruitment of a series of complement proteins produces a protein complex—the *membrane attack complex* (MAC)[1068]—and the resulting *complement cascade* can kill a pathogen, or activate an immune system phagocyte to destroy it.



Some research suggests severe COVID-19 is associated with higher ADCD—but lower ADCP. This points at the possibility that hospitalized patients are experiencing antibodies eliciting complement disposition rather more than phagocytosis—an indication that complement may be part of COVID-19 pathology through its contribution to inflammation-mediated tissue damage.[1069] Perhaps because it especially targets the RBD region of SARS-CoV-2's spike protein which is subject to mutations in variants, ADCD has shown greater loss of effectiveness against variants like Beta.[1070]

Antibody Fc Interactions Summarized

Most of the time Fc-FcR or Fc-Complement interactions work favorably for the host. For example, IgG effector function is governed by Fc-FcγR interactions. Critically, through the multitude of Fc-FcR interactions each individual creates distinct structural repertoires of IgG Fc domains. Thus, for instance, some people produce highly activating repertoires enriched for IgG1 and IgG3. Unfortunately, in rare instances a person's repertoire proves unfavorable and

[1068] On MAC in COVID-19, see Afzali et al., "The State of Complement," esp. 78 (Fig. 1).
[1069] Adeniji et al., "COVID-19 Severity Is Associated with Differential Antibody," 2, 5–7.
[1070] Richardson et al., "SARS-CoV-2 Beta and Delta Variants Trigger Fc Effector Function," 6.

the result is IgG Fc domain structure (e.g., reduced core fucosylation) that promotes deleterious outcomes rather than protective ones.[1071]

n-NAbs' Early Target Nucleocapsid (N) Protein

At the onset of SARS-CoV-2 infection, antibodies target the *nucleocapsid (N) protein*—the protein that binds to the inner RNA genomic core and provides it a protective shell. As we learned earlier in the book, the N protein is essential for the virus' replication and a part of the viral life cycle known as 'genome packaging,' whereby the replicated genome of the virus is placed into a protective envelope. N protein is highly *immunogenic*—it provokes an immune response.[1072] *Anti-N protein antibodies (IgA, IgM, and IgG)* have been found in the serum of COVID-19 patients and such antibodies show higher sensitivity and longer persistence than those with respect to other structural proteins of the virus.[1073]

A study in early 2020 employed saliva samples drawn from the back of the throat (*posterior oropharyngeal*), as well as blood samples (*serum*), and other sampling means, to measure SARS-CoV-2 viral load. The research involved 23 COVID-19 patients (13 male), ranging in age from 37–75 (median age 62). While the majority of this group experienced mild disease, ten experienced severe COVID-19, and two of them died. The study found a lower lymphocyte (B-cells and T-cells) count among those with severe disease, but not significantly so. Rising titers of anti-N protein IgM and IgG antibodies were found at 10 days or later after symptom onset was observed, and among a third of surviving patients measurable levels of antibodies were still present at 20 days. Detectable levels ('sero-positivity') were earlier for IgG than for IgM, and seropositivity at 14 days was higher for IgG (94%) than for IgM (88%). Notably, serum antibody levels were not correlated with clinical severity of COVID-19.[1074]

Another study, reported in 2021, examined 400 COVID-19 patients (confirmed by RT-PCR test), of whom 248 were admitted for hospital care, and of those 100 participated in a serology study. Of this last group, anti-N protein IgG was found in 55% at time of admission. The researchers also found that a high concentration of IgG against N-protein caused a threefold increase in risk of admission to the intensive care unit (ICU). They theorize that N-protein IgG may favor a higher inflammatory response and that its higher levels may be a reason for poorer outcomes in COVID-19.[1075]

Unfortunately, because such antibodies can also be found in prepandemic people—suggesting crossreactivity with other coronaviruses—the utility of serology results for COVID-19 may be diminished.[1076]

[1071] Chakraborty et al., "Symptomatic SARS-CoV-2 Infections Display Specific IgG Fc Structures," 2–3, 10.

[1072] For a brief general survey of studies on this targeting of the N protein, see Shah et al., "Overview of Immune Response," 5.

[1073] Zeng et al., "Biochemical Characterization of SARS-CoV-2 Nucleocapsid Protein," 618–19.

[1074] To et al., "Temporal Profiles of Viral Load"; see esp. 568–69, 72–73.

[1075] Batra et al., "Role of IgG Against N-Protein of SARS-CoV-2."

[1076] Dobaño, et al. "Immunogenicity and Crossreactivity of Antibodies."

B-Cell Produced Neutralizing Antibodies (NAbs)

One line of defense against SARS-CoV-2 and other coronaviruses is the body's development of *virus-neutralizing antibodies* (VnAbs), or more simply, *neutralizing antibodies* (NAbs). These are produced by B-cells in bone marrow. Virologist Ruta Kulkarni writes, "Neutralization occurs when antibody molecule(s) bind to the virus particle at its surface epitope and block viral attachment (to cellular receptor) and entry into host cell, and/or post-entry processes such as fusion and uncoating, thus resulting in loss of virion infectivity."[1077] Kulkarni points out that although scientists are unsure exactly how much antibody action is required to neutralize a virus, most think multiple antibodies must bind the virion to prevent infection. When immunoglobulin classes IgM, IgG, and IgA are all evident against SARS-CoV-2 then maximum neutralization activity against the virus is presumed as the antibodies work synergistically together.[1078]

Neutralizing Antibodies: Noteworthy Differences

NAbs against SARS-CoV-2 display various noteworthy differences.[1079]

	IgM	IgA	IgG
When Produced	within 7 days (day 4 on)	within 7 days (6–8 days)	10–14 days after infection
Course of Activity	rise 1st 7 days, persist 20–30, then diminish	peaks c. 20–22 days after symptom onset	peaks c. day 25
Stability of Antibody Titres	at least 5 months	rapid decline after 2 months	at least 5 months; up to 1 yr. plus
Relative Neutralization	highest	higher than IgG	lower than IgA
Differences in COVID-19	may peak earlier in mild cases	high levels associated with ARDS	higher neutralization levels in severe cases
Special Notes		about 5–10% of people show no detectable IgA after infection	

[1077] Kulkarni, "Antibody-Dependent Enhancement of Viral Infections," 10.
[1078] Pang et al., "Understanding Neutralizing Antibodies against SARS-CoV-2," 3.
[1079] Table content based on various sources, including Pang et al., "Understanding Neutralizing Antibodies," 3–4; Pisil et al., "Is SARS-CoV-2 Neutralized More Effectively by IgM and IgA than IgG Having the Same Fab Region?" Xiang et al., "Declining Levels of Neutralizing Antibodies," 6, and Hyeseon, et al. "Bispecific Antibodies Targeting Distinct Regions," 1–2.

IgM and IgA bear the early burden of neutralizing activity. Both are detected more frequently than IgG in the first two weeks after symptom onset, but IgG becomes prominent thereafter.[1080] It bears repeating that research confirms that *the highest neutralization activity against SARS-CoV-2 is achieved when all three immunoglobulins (IgM, IgA, and IgG) are known to be involved.*[1081] That given, we may still note various differences when the antibody types are compared.

Antibody IgM appears to be especially effective in neutralizing SARS-CoV-2. One study found that depletion from plasma of IgM compared to IgG and IgA, as measured by one indicator of its potency in inhibiting viral action (*half maximal inhibitory dilution* (ID50)), was significantly higher for *loss of neutralization activity* (see Table).[1082]

Depletion from Plasma	**IgM**	**IgG**	**IgA**
Using pseudoviral particles expressing S protein	5.5-fold decrease	4.5-fold decrease	2.4-fold decrease
Using 10 donor plasma samples exposed to live virus	4.0-fold decrease	2.8-fold decrease	no decrease

In most people IgM levels decline steadily over time and become undetectable, though in a very few they may persist at a detectable level for a year or more.[1083]

Above we observed that depletion of antibody IgA measured in human plasma (blood liquid without clotting) showed no decrease in overall neutralization activity. Another study, using human serum (blood liquid after clotting), reported that IgA neutralizing titres showed a decrease only when the initial levels had been high; medium and low starting levels did not show decrease.[1084]

Antibody IgG is the most persistent in its neutralizing activity. One study found that a year after symptom onset 90% of convalescing COVID-19 patients showed detectable levels (though only 43% still had neutralizing activity).[1085] IgG subclasses show differences in neutralizing activity. A study comparing samples from human serum found IgA1 and IgA3 are the subclasses that contribute the most activity, then IgA4, with IgA2 barely detectable.[1086]

[1080] Dispinsiri et al., "Neutralizing Antibody Responses," 3.
[1081] Mazzini et al., "Comparative Analyses," 8.
[1082] Gasser et al., "Major Role of IgM," 2. The figures are in reference to depleted plasma samples compared to non-depleted plasma.
[1083] Xiang et al., "Declining Levels of Neutralizing Antibodies," 6.
[1084] Mazzini et al., "Comparative Analyses," 7–8.
[1085] Xiang et al., "Declining Levels of Neutralizing Antibodies," 6.
[1086] Mazzini et al., "Comparative Analyses," 8.

Antibodies that contest the vial spike (S) protein are especially vital players. Microbiologist Yuhang Wang and colleagues observe, "The S protein is the major target for antivirus neutralizing antibodies and its binding to host cell receptor is critical for a productive infection and for cross-species transmission."[1087] In fact, 90% of NAbs in COVID-19 patients target the receptor binding domain (RBD) of S.[1088]

The Coronavirus Immunotherapeutic Consortium (CoVIC), with the aid of more than 50 research partners around the globe, has identified 370 antibodies against SARS-CoV-2's S protein.[1089] It has been estimated that anywhere from a 1,000 (10^3) to 100,000 (10^5) neutralizing antibodies are employed per spike protein in infected lung tissue. Although this may seem a very high ratio it is essential for ensuring enough of the spike protein is bound to by the antibodies.[1090]

Especially valued antibodies are those that bind the S proteins' *receptor binding domain* (RBD). In therapeutic interventions laboratory-engineered *monoclonal antibodies* (mAbs), which we'll discuss more later, can recognize the S protein's RBD in either its 'up' or 'down' configurations—or both. The following illustration shows such bindings.

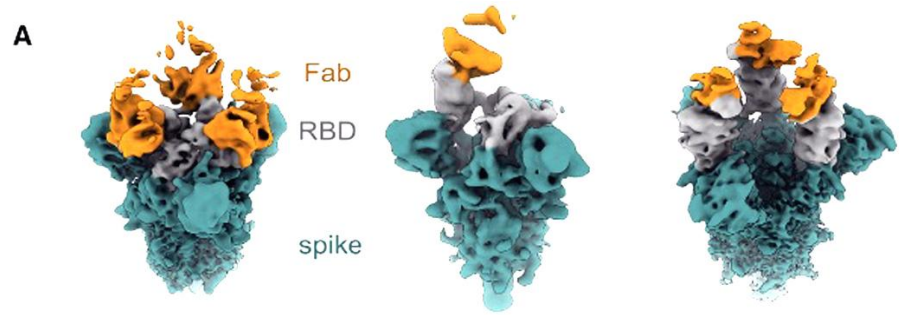
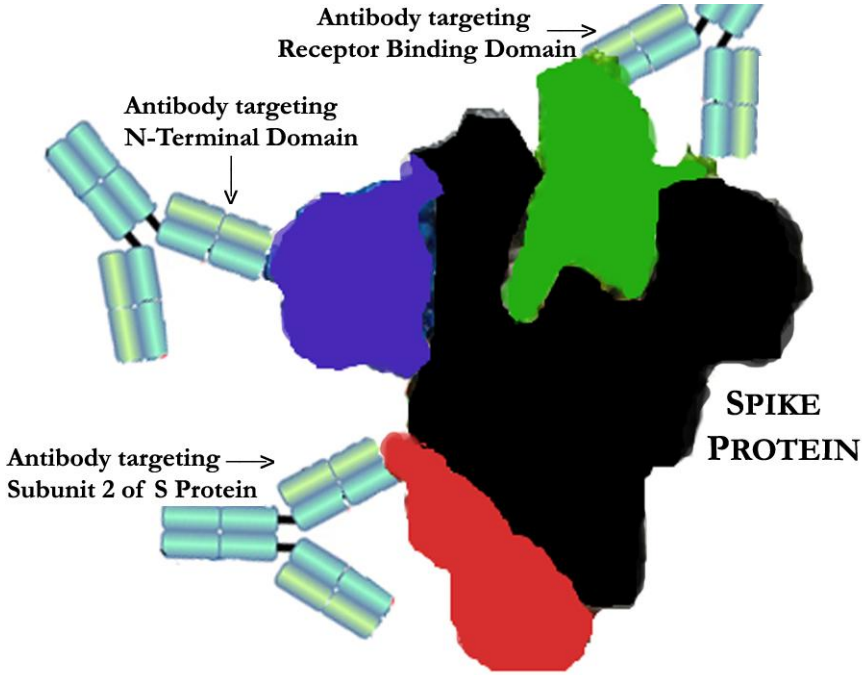


Figure 7. Determinants of binding, valency of interaction, and in vivo studies
(A) Cryo-EM Spike-Fab complexes showing different RBD conformations. The density for the Spike is shown in teal, the RBD in gray, and Fab in orange. Left: "all RBDs down" conformation with Fab 316 bound. Middle: "one RBD up" conformation with one Fab 158 bound. Right: "all RBDs up" conformation with 3 Fab 88s bound.

Illustration extracted from larger Figure 7 in Wanwisa Dejnirattsai, Daming Zhou, Helen M. Ginn, et al. "The Antigenic Anatomy of SARS-CoV-2 Receptor Binding Domain." *Cell* 184 (Apr. 15, 2021) 2183–2200. https://doi.org/10.1016/ j.cell.2021.02.032. Material is from page 2195 and is made available by Creative Commons Attribution (CC BY) License (https:// creativecommons.org/licenses/by/4.0/ legalcode). In addition to excerpting only a portion of the figure and its text, changes have been made to font type in the illustration's explanatory text.

[1087] Wang, Grunewald, and Perlman, "Coronaviruses," 7.
[1088] Niu et al., "A Structural Landscape of Neutralizing Antibodies," 2.
[1089] NIH, "Understanding SARS-CoV-2 Antibody Binding." Also see Hastie et al. "Defining Variant-Resistant Epitopes."
[1090] Sender et al., "The Total Number and Mass of SARS-CoV-2 Virions," 4.

The following illustration more specifically examines the S protein's structure and how antibodies may target it.



Antibodies target specific parts of the SARS-CoV-2 Spike Protein

As mentioned above, laboratory-engineered monoclonal antibodies (mAbs) are used as neutralizing antibodies against SARS-CoV-2. Their actions are briefly summarized in the following table (also related to the illustration above).[1091]

Target	Antibodies	Complex Formed	Intended Effect
NTD of S	mAbs	NTD/mAb	prevent conformational changes in the S protein and block membrane fusion and viral entry
RBD of S	mAbs, Nbs	RBD/mAb or RBD/Nb	prevent entry of SARS-CoV-2 into target cells

Key NTD = N-Terminal Domain; RBD = Receptor-Binding Domain
mAbs = Monoclonal antibodies; Nbs = Nanobodies

Generally, antibodies targeting RBD are more potent, but may also be more limited against variants than those that target NTD (which is more conserved).

[1091] Table based on Yang and Du, "SARS-CoV-2 Spike Protein: A Key Target," 1.

The nature of the infecting virus—its genetic sequencing—affects the breadth of the resulting neutralizing antibodies. A number of studies report that the DNA sequences of the genes encoding circulating antibodies reveal neutralizing antibodies (NAbs) are only a few mutations distant from their respective germline sequence. This observation suggests that in the germinal centers where so many B-cells develop there is an absence of the desired progression toward the best 'goodness-of-fit' (*affinity maturation*) needed to most effectively neutralize new variants of SARS-CoV-2. This also means a low capacity to induce the long-lived plasma cells (LLPCs) that generate large quantities of antibodies immediately after reinfection.[1092]

Thus, with each successive wave of the COVID-19 pandemic, neutralizing binding has changed. For example, when the binding of antibodies IgA and IgG to the original strain of SARS-CoV-2 was compared to their binding to the Beta variant the latter showed a significant decrease. SARS-CoV-2 *variants of concern* (VOCs), such as Beta, Delta, and Omicron, raise concerns because they show mutations to the spike (S) protein that help the virus evade immune system actions such as antibody neutralization. Specifically, in VOCs mutations occur in the very areas principally targeted by neutralizing antibodies—the receptor-binding domain (RBD) and the N-terminal domains (NTDs) of the virus. This is true both for those who are vaccinated and those who were previously infected. However, this does not mean the immune system has been disarmed; both T-cell function and spike-binding antibody levels still remain active. In fact, those antibodies able to bind outside of the places just mentioned are still facilitating potent antiviral functions that may confer protection from severe disease. Plus, antibodies are still using their Fc regions to drive effector functions such as those described earlier (ADCC and ADCT). Finally, to return to our example, antibodies like IgA and IgG that arose in response to infection by the Beta strain did better binding to it than those same kind of antibodies formed in response to an earlier strain.[1093]

In short, in the tug-of-war between virus and immune system, both sides are pulling. The immune system may occasionally falter and lose a step, but it is resilient. By the end of 2021, some research was showing that "antibodies evolve over time after SARS-CoV-2 infection to acquire increased potency and breadth."[1094] However, research also was indicating that the Omicron variant, with its 15 mutations of the virus' RBD, is highly successful escaping neutralizing antibodies (over 85% of 247 tested anti-RBD neutralizing antibodies).[1095]

[1092] Pecetta et al., "Antibodies, Epicenter of SARS-CoV-2 Immunology," 821.

[1093] Richardson et al., "SARS-CoV-2 Beta and Delta Variants Trigger Fc Effector Function," 1–2. Also see Grunst and Uchill, "Fc Effector Cross-Reactivity." On ADCC occurring at similar levels across types of SARS-CoV-2, see Yu et al., "Antibody-Dependent Cellular Toxicity Response to SARS-CoV-2," 4.

[1094] Hyeseon et al., "Bispecific Antibodies Targeting Distinct Regions of the Spike Protein," 10.

[1095] Cao et al., "Omicron Escapes … Majority of Existing SARS-CoV-2 Neutralizing Antibodies."

If Neutralization Fails: Antibody-Dependent Enhancement (ADE)

Antibody-binding to viral surfaces to prevent attachment and penetration serves as a primary response by the immune system. Absent successful neutralization (prevention of binding and entry) of SARS-CoV-2, the virus wins against the host. Regrettably, antibodies do not always succeed in sufficiently binding virus particles. What happens if the virus generally succeeds in getting past antibody neutralization? The short answer is: a lack of a neutralizing antibody response is a potent predictor of host death.[1096]

More broadly, failure in neutralizing activity can lead in rare instances to a phenomenon known as *antibody-dependent enhancement* (ADE).[1097] In ADE the antibodies intended to neutralize the virus S protein instead actually enhance viral infection—what has been called "traitorous behavior."[1098] This phenomenon has been observed in a number of serious viral diseases, including HIV and Ebola—and the severe human coronaviruses. ADE constitutes a major concern for those who treat viral illnesses—including vaccine-makers. The latter are concerned over the possibility, however remote, that the antibody response provoked by a vaccine could lead to an unintended consequence of ADE.

In viral infections, ADE may occur when an unfortunate confluence of events occurs involving a precipitating circumstance and taking opportunistic advantage of a biological pathway (as illustrated in this Table).[1099]

Precipitating Circumstance ⇨	n-NAbs bind to antigen without blocking or clearing infection	NAbs at sub-neutralizing levels
Pathway ⇨	enhanced antibody-mediated virus uptake into *Fc gamma receptor IIa* (FcγRIIa)-*expressing phagocytic cells* ⇩	excessive antibody Fc-mediated effector functions or immune formation ⇩
Outcome ⇨	increased viral infection and replication	exaggerated immune reaction: inflammation and immunopathology

At least one suspected case of ADE has been reported associated with COVID-19,[1100] but ADE is not only an extreme reaction but a rare one.

[1096] Dispinsiri et al., "Neutralizing Antibody Responses," 4.

[1097] For an overview of the subject, see Kulkarni, "Antibody-Dependent Enhancement." Also see Wen et al. "Antibody-Dependent Enhancement of Coronavirus."

[1098] Kulkarni, "Antibody-Dependent Enhancement," 9.

[1099] Table follows Lee et al., "Antibody-Dependent Enhancement and SARS-CoV-2," 1185, and Wang et al., "Antibody-Dependent Enhancement," 5. Cf. Ricke, "Two Different Antibody-Dependent Enhancement (ADE) Risks," 4–5.

T-Cells

Alongside antibodies, T-cells spring into action. This first orienting Table provides some perspective on their course of action and duration.[1101]

	Detected after Symptom Onset	Expansion Phase	Contraction Phase	Measurable Duration
CD4+	*7–10 days* (expected; but can be earlier)	*10–20 days* (after symptom onset)	*progressive* reduction after viral clearance (>1 mo. post infection)	*6–8 months +* (post infection)
CD8+	*7 days*	*10–20 days* (peaks at 14 days)	relatively more stable	

Delayed appearance (>15 days post symptom onset), and weak induction of T-cells is associated with severity of COVID-19.[1102] The overall quantity of T-cells is less important than their functionality; it looks to be superior in asymptomatic patients compared to those with even a mild case of COVID-19.[1103]

T-Cell Response against SARS-CoV-2 Proteins

T-cells, recall, recognize *major histocompatibility complex* (MHC) molecules (class I and II). CD8+ cytotoxic cells recognize MHC-class I molecules complexed with viral peptides and then destroy infected cells. CD4+ helper T-cells recognize MHC-class II molecules on antigen-presenting cells (APCs), following which they produce various cytokines in support of CD8 and B-cells.[1104]

CD4+ and CD8+ T-cells respond against most of SARS-CoV-2's proteins (e.g., S, M, N, ORF3a, nsp3, nsp4, and nsp12), but not equally. For example, against spike (S) protein, CD4+ responses dominate, most likely a reflection of their support of antibody generation. All together, more than 1400 potential epitopes (the place on an antigen molecule an antibody might attach to) have

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[1100] Ricke, "Two Different Antibody-Dependent Enhancement (ADE) Risks," 4; cf. Wen et al. "Antibody-Dependent Enhancement of Coronavirus," 486.
[1101] Table uses information from Bertoletti, Tan, and Le Bert, "The T-Cell Response to SARS-CoV-2," 3–4, and Moss, "The T Cell Immune Response against SARS-CoV-2," 186.
[1102] Bertoletti, Tan, and Le Bert, "The T-Cell Response to SARS-CoV-2," 3.
[1103] Bertoletti, Tan, and Le Bert, "The T-Cell Response to SARS-CoV-2," 6.
[1104] Bertoletti, Tan, and Le Bert, "The T-Cell Response to SARS-CoV-2," 1.

been identified. On the other hand, CD8+ is only weakly induced against nsp12.[1105]

One study found a clear hierarchy of CD4+ T-cell targets against SARS-CoV-2 (i.e., 'antigen specificity') and also a pattern for CD8+ T-cell targets in convalescing COVID-19 patients (N=20; Table below).[1106]

SARS-CoV-2 Protein	CD4+ T-cell target % (averages)	CD8+ T-cell target % (averages)
***Spike* (S)**	27% (other studies indicate about 66%)	~26% (other studies indicate about 50%)
***Membrane* (M)**	21%	22%
***Nucleocapsid* (N)**	11%	~12%
STRUCTURAL PROTEINS	(other studies indicate about 33%)	(other studies indicate about 36%)
***Nonstructural protein 3* (nsp3)**	~5%	--
***Nonstructural protein 4* (nsp4)**	~5%	--
***Nonstructural protein 6* (nsp6)**	--	15%
***Open reading frame 3* (ORF3)**	--	7%
***Open reading frame 8* (ORF8)**	~5%	10%

The chief take-away is recognizing T-cells address a wide array of targets. It may be worth noting that one study found most CD8+ T-cell responses coincided with a parallel CD4+ T-cell response, though with a smaller average magnitude (compared to the average peptide specific CD4+ T cell response).[1107]

T-Cells against the Spike (S) Protein

As the above illustration indicates, T-cell targets against SARS-CoV-2 focus on the spike (S) protein. This surface protein of the virus is key to viral attachment and entry into human host cells. SARS-CoV-2 S-protein reactive T-cell

[1105] Moss, "The T Cell Immune Response against SARS-CoV-2," 187, and Bertoletti, Tan, and Le Bert, "The T-Cell Response to SARS-CoV-2," 3.
[1106] Table based on data in Grifoni et al., "Targets of T Cell Responses," 1495.
[1107] Heide et al., "Broadly Directed SARS-CoV-2 Specific CD4+ T Cell Response," 8.

(both CD4+ and CD8+) responses are seen in COVID-19 at all levels of disease, but the amount of these reactive T-cells increases with disease severity.[1108] It is important to note, too, that spike-specific T-cell responses do not correlate with neutralizing antibody (NAb) quantities.[1109]

T-Cells against the Nucleocapsid (N) Protein

Although T-cells with respect to the spike (S) protein naturally receive great attention, we should also consider their address of the structural nucleocapsid (N) protein. Infection with betacoronaviruses (e.g., SARS and SARS-CoV-2) induces multi-specific and long-lasting T-cell immunity against the N protein. This makes sense because the N protein is abundantly produced during viral replication and T-cell response needs to remember that and be able to attack this protein. However, T-cells specific to nonstructural proteins (NSPs, e.g., NSP7 and NSP13) are preferentially detected in individuals who have not been infected by SARS-CoV-2 or had COVID-19. While the reason for this is not definitely known, some researchers suspect this occurs when people have been exposed to other coronaviruses and the immune system primes T-cells specific to the coronavirus ORF1 region which produces NSPs that are essential to building the structures inside the host cell needed for viral replication. Thus an innate system primed in this fashion can take preemptive action against viral replication getting started.[1110]

T-Cells against the Membrane (M) Protein

A third structural protein—the largest—is the membrane (M) protein. One study found that the highest frequencies of reactive CD4+ T-cells was against the M protein. M-reactive CD4+ T-cells expressed cytokines (IL-2, IFN-γ, and TNF-α) and effector molecules (granzyme B (GrzB), a serine proteinase).[1111] Another study found the most common CD4+ T-cell response was against peptides Mem_P30 and Mem_P36, both located in the N terminal domain of M protein.[1112]

CD4+ T-Cells

Upon exposure to MHC-class II molecules on antigen-presenting cells (APCs), which have upregulated co-stimulatory molecules that now deliver their signals, naïve CD4+ T-cells differentiate into different kinds of effector cells. They not only mediate help for B-cells and CD8+ T-cells, but also provide effector cells that migrate to sites of infection. These CD4+ T-cells activate APCs, release pro-inflammatory cytokines to recruit other immune cells to the site,

[1108] Thieme et al., "The SARS-CoV-2 T-Cell Immunity," 2.
[1109] Tan et al., "Rapid Measurement of SARS-CoV-2 Spike T Cells," 8.
[1110] Le Bert et al., "SARS-CoV-2-Specific T Cell Immunity," 462.
[1111] Thieme et al., "The SARS-CoV-2 T-Cell Immunity," 3.
[1112] Heide et al., "Broadly Directed SARS-CoV-2 Specific CD4+ T Cell Response," 8.

induce an antiviral state in surrounding cells, kill virus-infected cells, and some eventually become memory T-cells.[1113]

$CD4^+$ T-cells are most famous for their differentiation into 'helper' cells. As we saw in a previous chapter, $CD4^+$ helper cells have been divided into subsets, which include T-helper cell 1 (T_H1 or TH1), T-helper cell 2 (T_H2 or TH2), T-helper cell 9 (T_H9 or TH9), T-helper cell 17 (T_H17 or TH17), and, follicular helper T-cell (T_{FH} or TFH). These appear not to be equally represented in the fight against SARS-CoV-2, as the following Table indicates.[1114] (Interestingly, other research has found somewhat different results. For example, one study found T_H1 predominated in convalescing COVID-19 patients, with little to no T_H2 cytokine production.[1115])

$CD4^+$ subsets	T_H1	T_H2	T_H9	T_H17	T_{FH}
Actual against expected	lower	--	--	lower	higher*

*The elevated T_{FH} levels were found in hospitalized patients may be associated with the role of these cytotoxic cells, which can kill B-cells and dampen germinal center (GC) responses. It has been observed that the magnitude of cytotoxic T_{FH} response to the virus is greatest early in the course of infection and negatively correlated with antibody levels to SARS-CoV-2.[1116]

$CD4^+$ helper T-cells, following recognition of APC-presented antigen, produce various cytokines (IL-2, IL-21, Interferon gamma (IFN-γ), and Tumor Necrosis Factor–alpha (TNF-α)).[1117] T_H1 is especially associated with IFN-γ production, while T_H2 is associated with IL-4 production. Early induction of IFN-γ-secreting SARS-CoV-2-specific T-cells has been linked to milder cases of COVID-19.[1118]

$CD4^+$ regulatory T-cells (T_{regs}) have received comparatively little attention with respect to SARS-CoV-2. One study of hospitalized COVID-19 patients found reduced T_H1 and T_{reg} cells showed the strongest relation to severe disease.[1119] The reduction of T_{reg} cells matters because these cells help curtail excessive inflammation and cytokine storm. Thus there exists inverse cross-talking with $CD8^+$ T-cells, as shown in the following illustration.

[1113] For a review of these matters, see Swain, McKinstry, and Strutt, "Expanding Roles for $CD4^+$ T Cells in Immunity to Viruses," 136–37.
[1114] Meckiff et al., "Imbalance of Regulatory and Cytotoxic SARS-CoV-2-Reactive $CD4^+$ T Cells in COVID-19," 1350–51.
[1115] Grifoni et al., "Targets of T Cell Responses," 1496.
[1116] Meckiff et al., "Imbalance of Regulatory and Cytotoxic SARS-CoV-2-Reactive $CD4^+$ T Cells in COVID-19," 1350–51.
[1117] Bertoletti, Tan, and Le Bert, "The T-Cell Response to SARS-CoV-2," 1.
[1118] Kedzierska and Thomas, "Count on Us: T Cells in SARS-CoV-2 Infection," 1.
[1119] Caldrer et al., "Regulatory T Cells as Predictors of Clinical Course," 6–8.

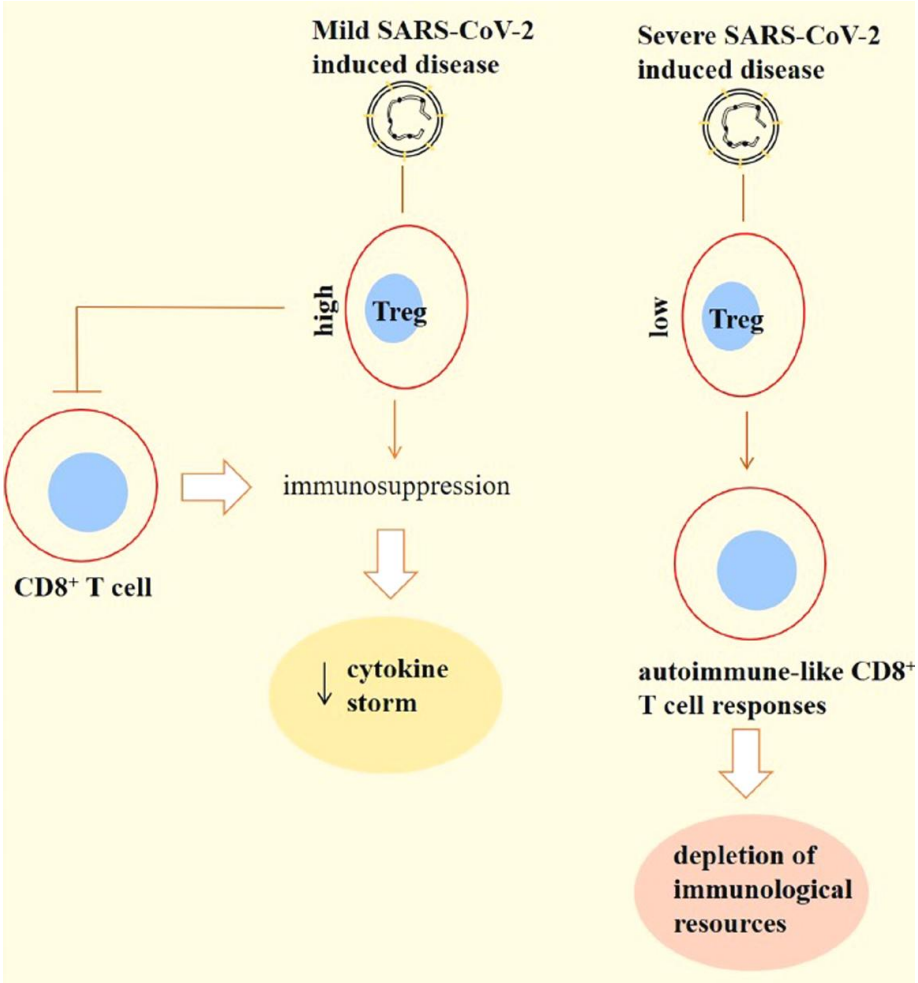


FIGURE 2 | Inverse cross talking between CD8+ T cells with regulatory T cells (Tregs) in patients with a SARS-CoV-2-induced disease. Tregs are high in patients with a mild disease. This will result in immunosuppression partly by suppressing the activity of CD8+ T cells. Such immunosuppression further reduces the extent of cytokine storm, thereby lessening the severity of the condition. By contrast, the number of Tregs is low in severe cases. This will cause responses from CD8+ T cells to self-antigens, and the cells are representing autoimmune-like states, which results in the depletion of immunological resources to combat against the virus. The outcome of this autoimmunity is higher viral persistence and a more severe condition.

Illustration on preceding page is from Mortezaee, Keywan, and Jamal Majidpoor. "CD8+ T Cells in SARS-CoV-2 Induced Disease and Cancer—Clinical Perspectives." *Frontiers in Immunology* 13 (Apr., 2022) art. 864298. doi: 10.3389/fimmu.2022.864298. Material is from page 8 and is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/ legalcode). Changes have been made in font size and type for explanatory text.

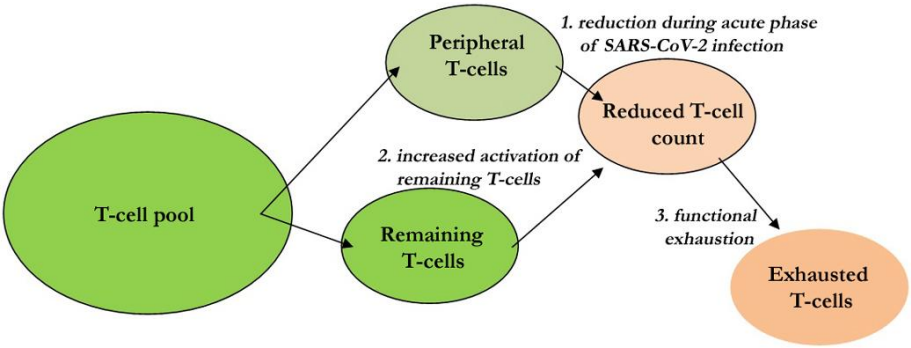
In a viral infection the innate system's dendritic cells (DCs) interact with CD8+ T-cells and thereby activate them to respond to specific antigen (Ag). This process, involving the formation of an immunological synapse (illustrated in the previous chapter) requires somewhat less than a day (18–24 hours). Then the activated T-cells begin to proliferate—and do so prodigiously. A cell cycle of 4–6 hours can see about a 10,000-fold expansion of their numbers, and there may up to 20 cell divisions in a week.[1120]

CD8+ T-cells can destroy virus-infected cells by rupturing their membrane, leading to dissolution (*lysis*), or through the secretion of antiviral cytokines (principally IFN-γ).[1121]

Both frequency and activation of CD8+ T-cells is associated with degree of severity in COVID-19 patients; they increase in severe disease. As the authors of one study put it, "The activation profile clearly distinguishes patients from healthy individuals. Patients who required hospitalization for COVID-19 demonstrated a significantly higher frequency of SARS-CoV-2–specific T cells and a more activated phenotype compared with patients with milder disease."[1122]

T-Cell Reduction and Possible Functional Exhaustion

A number of studies have found evidence of overall T-cell 'exhaustion' during the course of SARS-CoV-2 infection. It can be roughly mapped as follows:



While studies often find this overall pattern, they sometimes report different results with respect to CD4+ and CD8+, occasionally finding the former more reduced, and occasionally the latter more reduced. Notably, *peripheral T-cell depletion is associated with disease severity and mortality*. In the acute phase of COVID-19 viral load and T-cell reduction correlate with disease severity; in mild cases there is quick recovery while in severe cases the T-cell depletion continues, and lower peripheral T-cell counts are associated with higher mortality. Some evi-

[1120] Cui and Kaech, "Generation of Effector CD8+ T Cells," 1.
[1121] Bertoletti, Tan, and Le Bert, "The T-Cell Response to SARS-CoV-2," 1, and Boni, et al., "Degenerate CD8 Epitopes," 2.
[1122] Saini et al., "SARS-CoV-2 Genome-Wide T Cell Epitope Mapping," 3, 6, 10 (quote).

dence suggests that at least some exhausted T-cells may be sequestered rather than destroyed.[1123] In passing, it may be mentioned that not only are reductions in $CD4^+$ and $CD8^+$ associated with disease severity, but also other immune cells such as natural killer (NK) cells.[1124]

Affect of SARS-CoV-2 Variants

Overall, research affirms that the mutations found in common SARS-CoV-2 variants (e.g., Alpha, Beta, Delta, Omicron) display a negligible impact on T-cell (CD4+) reactivity, probably because CD4+ T-cells target highly conserved and thus non-mutating regions of the spike (S) protein.[1125] In fact, research has revealed that some immunodominant CD8 T cell epitopes are located within highly conserved SARS-CoV-2 regions that cannot mutate without impairing SARS-CoV-2 functionality (see illustration, next page).[1126]

Early in the study of the Omicron variant, T-cells emerged as a significant line of defense compared to antibody response. Because of Omicron's high number of mutations on the spike (S) protein of SARS-CoV-2, the variant showed a high escape from neutralizing antibodies. Since vaccines target this viral protein and aid antibody response this same escape tendency affects vaccine effectiveness. However, research reported from South Africa showed that 70–80% of the CD4 and CD8 T-cell response to spike was maintained—a response similar to that found against earlier Beta and Delta variants.[1127] Moreover, whether the result of actual infection or by virtue of vaccination the majority of T-cell responses cross-recognize the Omicron variant.[1128]

Booster shots of vaccine help, even though a normal full-course of two shots is good. One study, focused on patients with multiple sclerosis (MS), found that 6 months after a second dose the memory T-cell responses to variants Delta and Omicron were comparable to those in healthy adults. But a booster shot increased the frequency of $CD8^+$ cytotoxic T-cells, and did so irrespective of variant.[1129]

The truly good news is, as one team of researchers wrote, "even in the event of abrogation of specific CD8 T cell responses directed against epitopes that have been inactivated as a result of SARS-CoV-2 variant mutations, the evidence that CD8-mediated responses are not only widely multi-specific but can also target highly conserved (and potentially invariable) spike sequences makes mutation-mediated evasion from the overall protection afforded by CD8 T cells most unlikely."[1130]

[1123] Shrotri et al., "T Cell Response to SARS-CoV-2 Infection," 6–8 (upon which the illustration is based), and 12.
[1124] Li et al., "Elevated Exhaustion Levels," 8.
[1125] Mazzoni et al., "SARS-CoV-2 Spike-Specific $CD4^+$ T Cell Response," 3–4.
[1126] Boni, et al., "Degenerate CD8 Epitopes," 1.
[1127] Keeton et al., "SARS-CoV-2 Spike T Cell Responses," 2.
[1128] Keeton et al., "T Cell Responses to SARS-CoV-2 Spike Cross-Recognize Omicron."
[1129] Madelon et al., "Omicron-Specific T-Cell Responses after a Third Dose," 401.
[1130] Boni, et al., "Degenerate CD8 Epitopes," 8.

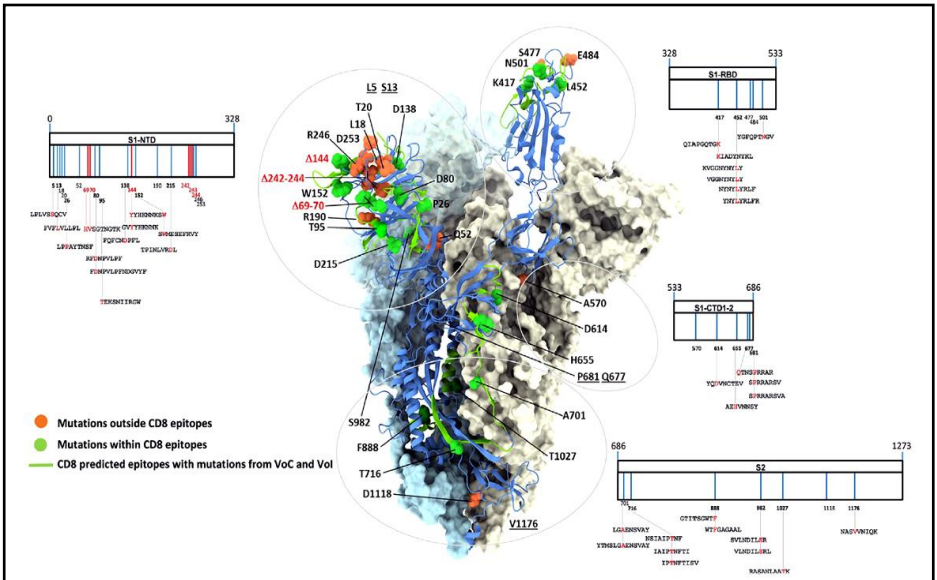


FIGURE 1 | SARS-CoV-2 spike mutation and deletion map of the prominent circulating VoC/VoI and SARS-CoV-2-specific CD8 T cell epitopes containing mutations. SARS-CoV-2 spike organization: each vertical line within the external charts indicates individual amino acid mutations or deletions of the UK, South Africa, Brazil, Nigeria, California, New Yorker and India variants (in blue and red, respectively) among the S1 and S2 subunits and the Receptor Binding Domain (RBD). SARS-CoV-2-specific CD8 T cell epitopes within the spike protein described in the literature and containing variant mutations are depicted at the bottom of each chart. All amino acid sequences of the 35 CD8 T cell epitopes are reported; mutation and deletion sites are indicated by their position number (numbers in bold indicate mutations contained in described epitopes) and are depicted in red within the epitope sequences. The trimer structure of a spike ectodomain (PDB 6ZXN), with one protomer in blue cartoon (RBD-up) and the two other protomers (both RBD-down) represented as light blue and ice surfaces, is shown as a reference in the central part of the figure. Mutation-affected residues outside or within CD8 epitopes are represented as orange and green spheres, respectively. Spike secondary structure elements containing CD8 epitopes affected by variant mutations are shown as green ribbons. Specific amino acids located within unresolved regions or targeted by deletions are underlined or shown in red, respectively. Glycan-modified regions as well as the NTD surface of one spike protomer were omitted for clarity.

Illustration is from Carolina Boni, Davide Cavazzini, Angelo Bolchi, Marzia Rossi, et al. "Degenerate CD8 Epitopes Mapping to Structurally Constrained Regions of the Spike Protein: A T Cell Based Way Out from the SARS-CoV-2 Variants Storm." *Frontiers in Immunology* 12 (Sept., 2021) art 730051. doi: 10.3389/fimmu.2021.730051. Material is from page 3 and is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/legalcode). Changes have been made in font size and type for explanatory text.

Cross-Reactivity

Cross-reactivity (also known as *heterologous immunity*)—the occurrence of an antigen binding to an antibody specific to a different antigen—is a phenomenon observed with T-cell response to SARS-CoV-2. The human body may be 'primed' to resist SARS-CoV-2 infection by having been previously infected by a human coronavirus. This may happen because *T-cell epitopes* (the part of the viral antigen an antibody binds to) may be shared across coronaviruses. Thus, when SARS-CoV-2 infection occurs, the host's antibodies are able to recognize antigenic macromolecules and bind. For example, one study looked at 20 people unexposed to SARS-CoV-2 against a comparable group of 20 COVID-19 patients and found half of the unexposed showed non-spike-specific $CD4^+$ T-cells. All of them had seropositivity to endemic coronaviruses HCoV-OC43 and HCoV-NL63.[1131] Not only can preexisting cross-reactive $CD4^+$ T-cells enhance immune responses when SARS-CoV-2 infection occurs, but they also appear to enhance mRNA (Pfizer's) vaccination response.[1132]

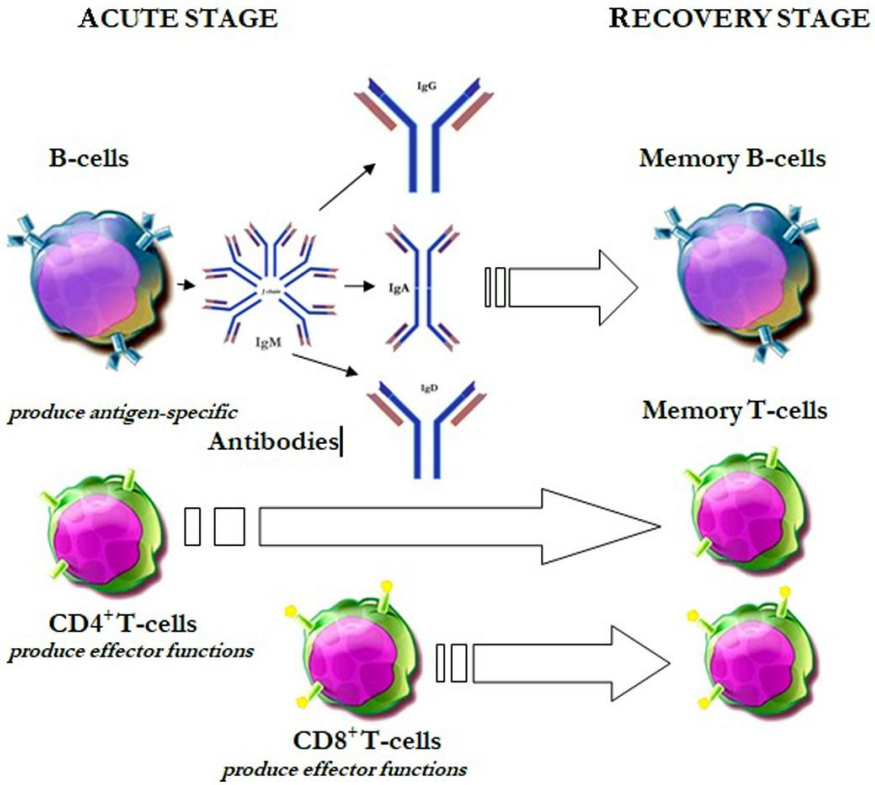
Presumably, this provides the host with an extra measure of protection, although some evidence suggests that cross-reactivity may also contribute sometimes to poorer outcomes. In one sizable study of people across the age spectrum, cross-reactive antibodies were found for both SARS-CoV-2's spike (S) protein (its receptor-binding domain (RBD)) and its nucleocapsid (N) protein, though in each case in modest numbers—0.9% against RBD of S protein and 16% against N protein. However, it seems that cross-reactivity may be seen more often in children and young adults, perhaps because these populations are more likely to have experienced a recent coronavirus infection. [1133] Overall, the existing evidence supports the idea that a degree of cross-reactivity of T-cell responses between human coronaviruses may be common.[1134]

T-Cell Response Compared to B-Cell Antibody Response

While T-cell responses are occurring alongside B-cell generated antibodies, a discord between virus-specific antibody levels and T-cell responses has been observed. Some COVID-19 patients—noticeably those either asymptomatic or with mild infections—may show multi-specific T-cells to SARS-CoV-2, but an absent expected antibody response. Also noted in recovering COVID-19 patients have been cases of different levels of neutralizing antibodies (NAbs) though similar quantity of SARS-CoV-2-specific T-cells.[1135]

[1131] Grifoni et al., "Targets of T Cell Responses," 1494.
[1132] Loyal et al., "Cross-reactive $CD4^+$ T Cells Enhance SARS-CoV-2 Immune Responses."
[1133] Moss, "The T Cell Immune Response against SARS-CoV-2," 188. The specific percentages of patients showing cross-reactivity is from Ng et al., "Heterologous Humoral Immunity," 2.
[1134] Shrotri et al., "T Cell Response to SARS-CoV-2 Infection," 9.
[1135] Bertoletti, Tan, and Le Bert, "The T-Cell Response to SARS-CoV-2," 3.

Measuring adaptive immune system success against SARS-CoV-2 requires first seeing what recovery looks like from the standpoint of the immune system. This can be simply illustrated through a contrast of the active (i.e., 'acute') state of infection and afterwards ('recovery').



The adaptive immune system has 'adapted' to the reality of the danger of SARS-CoV-2 by first 'effecting' actions against the active virus, then 'remembering' what worked for the future. Thus the recovery stage is a changed system with stored memory cells ready for any reoccurrence of the virus.

The above illustration remains focused on the adaptive immune system. However, we are at a point where we can profit from again taking a step back to examine both the innate and adaptive immune systems as they work in a coordinated effort to combat invasion by SARS-CoV-2. We may thus conclude this section by examining an illustration of this entire immune response and coordinating it with our interest in the next section, which is attempting to measure the success of that response. In other words, the following illustration looks at the immune response in terms of disease progression and levels of severity.

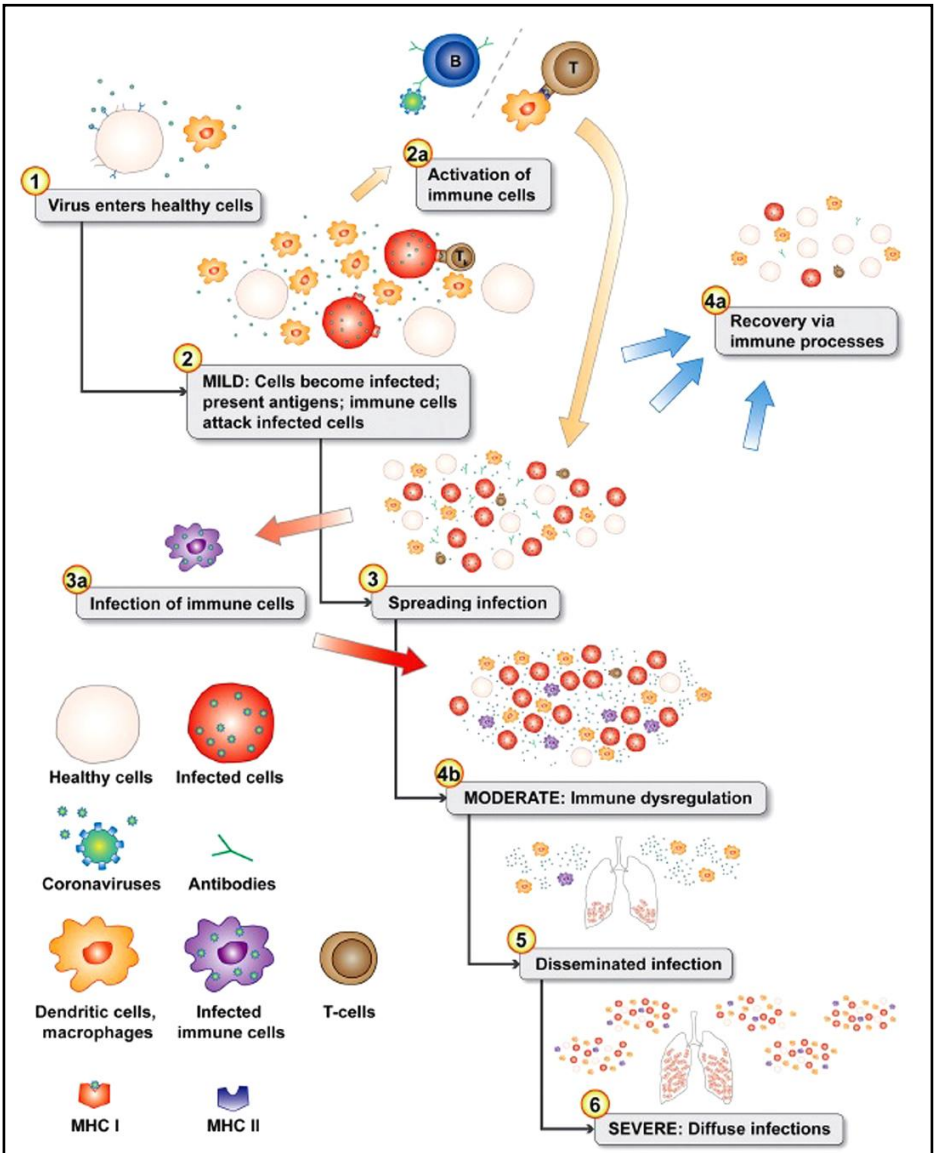


FIGURE 2 | Disease progression model with normal immune responses during the initial mild symptoms phase (see 1–3). Antigen presenting cells migrate to the lymph nodes to activate T cells (2a). The progression gate to moderate and server disease is the infection of phagocytic immune cells (3a) leading to immune dysregulation (4b). In the lungs, chemokines attract additional dendritic cells and immature macrophages that are subsequently infected in a positive feedback-loop infection cascade (4b). Infected phagocytic immune cells disseminate throughout the body infecting additional organs (5 & 6). Levels of chemokine and cytokines in the lungs from infected cells can create a cytokine storm (6).

Preceding illustration from Darrell O. Ricke. "Two Different Antibody-Dependent Enhancement (ADE) Risks for SARS-CoV-2 Antibodies." *Frontiers in Immunology* 12 (Feb., 2021) art. 640093. doi: 10.3389/fimmu.2021.640093. Material is from page 4 and is made available by Creative Commons Attribution (CC BY) License (https:// creativecommons.org/licenses/by/4.0/legalcode). Changes have been made to font type in the illustration's explanatory text and one grammatical correction ("an positive" to "a positive" has been made).

The illustrations work well as an overview, but the evidence from clinical studies show immune system responses are quite varied among people—they are *heterogeneous*. To make better sense of what actually happens we need a model that sketches in broad strokes this diversity of experience. Immunologists Alessandro Sette and Shane Crotty have taken steps to create such a model for us to profit from in understanding how immune system response correlates with degrees of disease severity. The following Table is based on their work.[1136]

	Asymptomatic/ Mild/Moderate	**Severe/Critical**
Innate Immune Response (I)	'Normal' response is within hours of infection. Little, if any, delay occurring means virus is hindered and adaptive immune system is primed by alarms.	Delayed and impaired Type I and Type III IFN response results from successful viral evasion..
Viral Replication	Replication is delayed, limited, contained by effective response.	Initial replication unabated and successful in upper respiratory tract and lungs. Viral burden is high.
Innate Immune Response (II)	No 'doubling-down' is required as initial response is sufficient.	'Doubling-down' by an ever-expanding innate response marked by massive numbers of neutrophils in the lungs. Elevated risk of cytokine storm.
Adaptive Immune Antibody Response	Antibody response relatively rapid (about 6–10 days after priming) and controls infection.	Antibody response is delayed.
Adaptive Immune T-Cell Response	T-cell response relatively rapid and controls infection. Specific T-cell responses significantly associated with milder disease.	T-cell response is delayed. Late adaptive T-cell responses *too late*.

The key lesson here is simple and graphic: *the response time of the innate immune system significantly impacts the effectiveness of the adaptive immune system response*.

[1136] Sette and Crotty, "Adaptive Immunity to SARS-CoV-2 and COVID-19," 862, but see entire article for details and elaboration. (Any deficencies in the Table should be charged to me.)

Tests and Objective Markers

From the standpoint of human experience the measurement of immune system success against COVID-19 is 'feeling better'—a subjective sense of recovery. But our interest here is in objective markers that measure such success by the body—or indicate complications with recovery. Although a great many such markers have been proposed for clinical use—many of them touched upon earlier as we reviewed matters—here we will focus briefly only on a few.

Various ways to measure post-infection immune system response to SARS-CoV-2 include:

- *enzyme-linked immunosorbent assay* (ELISA) *tests* detect and measure antibodies in blood;
- *enzyme-linked immunospot* (ELISpot) *assays* measure the frequency of cytokine-secreting cells at the single cell level;
- *serology tests* and *serologic assays* (covered in the previous chapter) are the most commonly used measurement tool;
- *T-cell count* (aka *thymus-derived lymphocyte count*) drawn from a blood sample;
- *T-Detect COVID-19 test* is used to identify T-cells that recognize SARS-CoV-2 antigens.

The most relied upon of these midway through 2022 remained antibody testing, though increasing attention was being paid to T-cell testing.

General Measures of White Cells

In a study of 30 hospitalized COVID-19 patients, although the total number of white blood cells (WBCs) was comparable to healthy people, some significant differences were found when looking at particular kinds of WBCs. Thus, circulating lymphocytes were found to be *reduced* compared to healthy people—and this was true for all subtypes (B, T, and NK). So, too, were circulating eosinophils and basophils. On the other hand, neutrophils were *higher*.[1137]

Antibody Measures

Both kinds of measurement discussed at the end of the previous chapter (serology tests and serologic assays) relate to protection against symptomatic COVID-19 infection, so detecting the presence of antibodies suggests protection—'immunity' (a matter discussed at length later in the chapter).

But not everyone produces antibodies against SARS-CoV-2 that are later detectable. Just how many may not is still a little uncertain. Typically, IgG antibodies, which persist the longest, are measured (using RT-PCR tests). The more severe the case of COVID-19 the higher the levels of IgG that can be expected when later testing occurs. But loss of *seropositivity* (i.e., the detectable presence of the antibody in blood) over time complicates assessing matters. For example,

[1137] Mazoni et al., "Impaired Immune Cell Cytotoxicity," 4698–99.

one study of 2,547 persons confirmed to have had a SARS-CoV-2 infection found 6.3%—about 1-in-16 persons—were *seronegative* (i.e., had no detectable presence of IgG). Of the rest, seronegative rates increased across a timeframe from 2 weeks to 90 days post symptom onset.[1138] Another study reported that the probability of continuing to find seropositivity declined significantly over time—from 98.6% (after 1 week) to 74.3% (after 2 months).[1139]

As reported by the CDC in late 2021, "The vast majority of persons with SARS-CoV-2 infection generate detectable anti-SARS-CoV-2 antibodies, with multiple studies reporting seroconversion rates of 90% or higher." [1140] But that finding might be qualified by noting application among those with 'mild or moderate' disease.[1141] More pertinently still, perhaps, is the analysis that found studies reveal that "seroconversion rates are surprisingly variable."[1142] Perhaps, then, the best way to characterize the situation is along the lines presented by Jéromine Klinger and colleagues:

> SARS-CoV-2 spike-, RBD- and nucleocapsid-specific serum and plasma Abs of IgM, IgG, and IgA isotypes are found in most COVID-19 patients, with neutralizing activities developing within two weeks of infection and declining over time. However, the neutralizing titers vary greatly and correlate with Ab binding levels against RBD, spike, and/or nucleocapsid, and with age, symptom duration, and symptom severity.[1143]

Also worth noting, as the CDC report points out, a number of factors contribute to the *degree* of immune response. One significant factor is the severity of the infection; for both binding and neutralizing antibodies the antibody titers rise more quickly and reach a higher peak the more severe the infection is.[1144]

Indeed, different tests to measure immune system response can be vitally important in *diagnosing the severity of disease infection*. For example, a systematic review and meta-analysis published early in 2022 examined 145 studies, with 26 immunological, 11 hematological, 5 inflammatory, 4 coagulation, and 10 biochemical variables reported. It found significant correlations between levels of certain variables and severity of illness. The profile for increased severity and mortality features the 'cytokine storm' (*hypercytokinemia*) discussed earlier in this volume. Cytokines (e.g., IL-1β, IL-1Ra, IL-2R, IL-4, IL-6, IL-8, IL-10, IL-18, TNF-α, and IFN-γ), many of which we have encountered more than once in describing the war against pathogens, promote inflammation and inflammation out-of-control is like a programmed fire intended to thin brush instead burning down the entire forest. Contrasting this profile is another, for those who survive

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[1138] Petersen et al., "Lack of Antibodies," e3071.
[1139] Kaufman et al., "Insights from Patterns of SARS-CoV-2 Immunoglobulin G."
[1140] CDC, "Science Brief: SARS-CoV-2 Infection-Induced and Vaccine-Induced Immunity."
[1141] Pisil et al., "Is SARS-CoV-2 Neutralized More Effectively by IgM and IgA than IgG Having the Same Fab Region?" 1.
[1142] Liu, "Predictors of Nonseroconversion After SARS-CoV-2 Infection," 2454.
[1143] Klinger et al., "Role of IgM and IgA Antibodies in the Neutralization of SARS-CoV-2," 4.
[1144] CDC, "Science Brief: SARS-CoV-2 Infection-Induced and Vaccine-Induced Immunity."

the war with less severe illness. For them, biological markers (e.g. higher levels of eosinophils, monocytes, NK cells, B-cells, and T-cells (both $CD4^+$ and $CD8^+$)) show functional innate and adaptive immune responses.[1145]

However, it is important not to assume that general profiles apply to every individual who contracts COVID-19. Infection by SARS-CoV-2 has led to some counterintuitive findings from the start. Antibodies include immunoglobulin M (IgM), the largest antibody; immunoglobulin A (IgA), which constitutes about 10–15% of the body's antibodies; and immunoglobulin (IgG), the most common antibody. Typically, following infection testing of antibody presence in the blood shows IgM levels rising, then falling, while IgC levels steadily increase. But with COVID-19 this normal picture doesn't seem to hold with any regularity. In various studies IgM has been found to increase before IgG, after IgG, simultaneously or even not at all.[1146]

T-Cell Measures

T-cell counts have been supplemented by the development of the *T-Detect COVID assay*. Designed to be a simple alternative to antibody tests, it attained 97.1% sensitivity (defined as positive percent agreement) and 100% specificity (defined as negative percent agreement) as compared with PCR testing from 15 days or more after diagnosis.[1147] Differences have been found between children and adults, with total IFN-γ $CD4^+$ and $CD8^+$ T-cell responses against SARS-CoV-2 structural proteins (E, M, and N) significantly lower in children (perhaps due to differences in prior immunity to seasonal human coronaviruses).[1148] Some research has found that SARS-CoV-2-specific memory T-cell responses can be maintained in COVID-19 convalescent patients 10 months post infection regardless of disease severity.[1149]

Final Note: Recovery Period & Complications

Recovery from COVID-19 is not a uniform process that is experienced by all people in the same manner. Studies suggest that even after a COVID-19 patient is no longer symptomatic—is 'convalescent'—their immune system still remains active in ways that indicate complete restoration from infection by SARS-CoV-2 may require a longer time than is true for other pathogens.[1150] For example, in some patients—especially those with pre-existing cardiovascular risk—a prolonged overactive state of the immune system may be related to endothelial dysfunction (i.e., instead of opening in a normal manner the heart's surface large blood vessels constrict), which can lead to increased expression of inflammatory cytokines.[1151]

[1145] Qin et al., "Identification of Parameters Representative of Immune Dysfunction."
[1146] Ryding, "What Is Seroconversion?"
[1147] Sheridan, "COVID-19 Testing Turns to T Cells," 534. See Dalai et al., "Clinical Validation."
[1148] Cohen et al., "SARS-CoV-2 Specific T Cell Responses," 11.
[1149] Jung et al., "SARS-CoV-2-Specific T Cell Memory," 7.
[1150] Zhang et al., "Adaptive Immune Responses to SARS-CoV-2," 8.
[1151] Chioh et al., "Convalescent COVID-19 Patients," 10.

Vitamins and supplements like zinc are often recommended by health professionals—one area where anti-vaxxers and medical professionals actually might agree. *Vitamin C* (ascorbic acid) is perhaps the most popular vitamin people take as a supplement to their diet. It is considered an essential nutrient and is important to the body in many ways, including boosting the immune system. *Vitamin D* is probably most famous for its role in bone health, but is also thought to benefit the immune system by increasing antimicrobial peptides the body uses as a natural antibiotic and to be valuable in protecting against respiratory infections. *Zinc*, too, supports the immune system, as well as boosting metabolic function. Thus all three are beneficial when taken in appropriate amounts.

Vitamin D

Of the three, vitamin D has the best support for possessing value against COVID-19 infection. As a vitamin it is unique in being a fat-soluble steroid hormone. Of its two forms—D_2 and D_3 (*calcitriol*)—the latter has a long half-life in circulation and also shows greater affinity for the circulating vitamin D binding protein. Its role in immune system function is long established. It modulates expression of about 5% of human genes—including many related to immune system response. In that respect, vitamin D appears to have more impact on the severity of virus infection than on the likelihood of being infected.[1152]

More specifically, the vitamin D receptor (VDR) on various cells of the human innate immune system (e.g., B- and T-cells) bind to vitamin D_3 (calcitriol) to form a *calcitriol-VDR complex* that facilitates expression of anti-microbial compounds (peptides like cathelicidins, defensins, IL-37) which act in various ways to defend host cells. Vitamin D also acts on adaptive immunity by modulating cytokines and controlling inflammatory response mediated by Th1 lymphocytes. This latter regulating function appears compromised by vitamin D deficiency.[1153]

The principal circulating form of Vitamin D in blood is *serum 25-hydroxyvitamin D* (25(OH)D), and measuring its level is widely regarded as the best indicator of vitamin D supply to the body. A normal range is 25–80 nanograms per milliliter (ng/mL). An Israeli retrospective study published in early 2022 used this measure to study COVID-19 patients with a positive PCR-confirmed infection and whose 25(OH)D levels prior to infection had been documented. These parameters resulted in 253 patients for the period April 7, 2020–February 4, 2021. The researchers found that lower levels (<20 ng/mL) of 25(OH)D were associated with more severe cases of COVID-19. Among se-

[1152] Rhodes et al., "Perspective: Vitamin D Deficiency."

[1153] Munshi et al., "Vitamin D Insufficiency," 736; Chiodini et al., "Vitamin D Status and SARS-CoV-2 Infection," 2. For more specialized examination, see Speekaert, De Buyzere, and Delanghe, "Vitamin D Binding Protein."

vere cases 87.4% had such low levels, though more than a third (34.3%) of moderate cases also had these low levels. Compared to patients whose levels prior to infection had been solidly in the normal range (≥40 ng/mL), those with low levels were 14 times more likely to have severe or critical COVID-19.[1154]

Research at the University of Chicago Medicine found that having vitamin D levels above those usually regarded as sufficient (30 ng/ml) may lower the risk of COVID-19 infection, especially for African-Americans. Levels of 40 ng/ml or greater seemed to decrease risk of infection. Moreover, as noted in a previous chapter, vitamin D deficiencies (<20 ng/ml) seem associated with greater vulnerability to COVID-19 among African-Americans, but not for Whites.[1155] Since African-Americans as a group are more reticent about receiving medical care, this information might be especially useful for them.

Vitamin D as a dietary supplement is recommended at 600–800 IUs a day, with most people tolerating up to 4,000 IUs a day, but over 10,000 is probably unsafe. And before anyone rushes out to stockpile it, notice should be taken that a genetic study undertaken to eliminate the kind of confounding variables that make studies like the one done at the University of Chicago Medicine hard to test for verifying results found no evidence to suggest that taking vitamin D supplements would help against COVID-19.[1156]

This conclusion has been echoed by other groups studying the matter. For example, in the United Kingdom the National Institute of Health Care Excellence (NICE), Public Health England (PHE), and the Scientific Advisory Committee on Nutrition (SACN) all concurred that taking a vitamin D supplement in autumn and winter is desirable and that low vitamin D levels are associated with more severe cases of COVID-19. But they cautioned there remains insufficient evidence that vitamin D is sufficient to prevent or treat COVID-19.[1157]

This was further corroborated by a comprehensive systematic review and analysis of 26 studies that was published in early 2022. It examined vitamins C and D, and zinc with respect to COVID-19. Vitamin C showed no significant effect on either intubation or mortality. Zinc supplementation also showed no significant reduction in mortality. The best news was with vitamin D. Although vitamin D supplementation did not reduce the risk of mortality, it was associated with a significant reduction in intubation and a reduced length of hospital stay. But the study cautioned such benefits were by no means certain.[1158]

There is no evidence to date suggesting that vitamins C or D, or zinc, can either prevent or treat COVID-19. Moreover, it is possible to overdose with

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[1154] Dror et al., "Pre-Infection 25-Hydroxyvitamin D_3 Levels and Association with Seveirty of COVID-19." See the Discussion, p. 10, for specifics on matters such as increased risk of infection and association with the cytokine storm leading to ARDS.

[1155] Caldwell, "Study."

[1156] Butler-Laporte, et al., "Vitamin D and COVID-19."

[1157] Wise, "COVID-19: Evidence Is Lacking."

[1158] Beran et al., "Clinical Significance of Micronutrient Supplements." See, esp., pp. 4–5, 8.

something like zinc, inducing headache, nausea and vomiting, and other unpleasant consequences—and all without achieving either any protection from COVID-19 or curing it. That said, pro-vaccine advocates have no argument against the appropriate use of supplements; they only object to misleading claims about them or the abuse of them.

Some Treatment Candidates

Researchers continue to examine alternative ways to prevent and treat COVID-19. Among things investigated are the following:

- antibody treatments;
- anti-inflammatories (e.g., colchicine);
- antivirals (e.g., hydoxychloroquine, remdesivir, etc.);
- *cannabidiol* (CBD);
- inhibitors; and
- naturopathic and homeopathic alternatives.

As is true with many matters discussed in this volume there may be overlap among categories. But these labels serve as useful broad indicators of approaches being explored. Here we only touch upon these different matters.

Antibody Treatments

As we have seen, antibodies play a major and key role in the war against SARS-CoV-2. The following table provides an overview.

Treatment	Description
Passive Immunization	The antibodies produced in one person's immune system are introduced into another person's immune system to provide immediate but temporary protection.
Engineered Antibodies	Antibodies made in a laboratory; there are five major kinds (antibody-drug conjugates (ADCs), bispecific (BsAb), recombinant, and therapeutic (esp. monoclonal (mAbs), but also polyclonal (pAbs)).
Vaccination	An indirect antibody treatment in that vaccines provoke the body to respond *as if* the virus was fully present by producing antibodies against it.

Passive immunization is a technique known for more than a century and used for temporary protection by extracting antibodies produced in one person's body (someone who has successfully survived an infection) and transferring them to another person. Against SARS-CoV-2, IgM and IgG antibodies from recovered COVID-19 patients offer an opportunity to increase neutralizing anti-

bodies (NAbs) in at-risk populations such as the elderly. This therapeutic approach has been employed since the early days of the pandemic. When it was used in countries such as China and Korea.[1159] A common approach to passive immunization therapy is the use of *convalescent plasma* (CP). COVID-19 convalescent plasma (CCP) is regarded as safe and possessing high potency.[1160] Near the end of 2021 emergency use authorization was granted in the United States for CCP. In an EUA Fact Sheet the following explanation was provided:

> COVID-19 convalescent plasma is the liquid portion of blood from donors who have recovered from COVID-19. The blood from people who recover from COVID-19 contains substances called antibodies, which are capable of fighting the virus that causes COVID-19. For some other diseases caused by respiratory viruses, giving people the liquid portion of blood that contains these antibodies, called plasma, obtained from those who have recovered from the virus, may lead to more rapid improvement of the disease. Patients with COVID-19 and weakened immune systems may improve faster if they receive plasma from those who have recovered from COVID-19, because it may have the ability to fight the virus that causes COVID-19.[1161]

Engineered antibodies include a variety of technologies. Antibody-drug conjugates (ADCs) use chemical linkers to bind other molecules to the antibody to produce a modified antibody. Bispecific antibodies (BsAbs) combine within a single molecule the antigen-binding sites of two antibodies (hence the 'bi-'), thus providing binding advantages against infectious agents. Recombinant antibodies are antibody fragments and have been looked at therapeutically because they possess some advantages over monoclonal antibodies. With respect to COVID-19 it is the therapeutic *monoclonal antibodies* (mAbs) that receive great attention. A principal target of such neutralizing antibodies is the receptor binding domain (RBD) of SARS-CoV-2's spike (S) protein.[1162] Hundreds of mAbs against the S protein exist, and many specifically recognize RBD in either its 'up' or 'down' configuration, or both. Potent mAbs bind close to where the RBD interact with ACE2 receptors and block that interaction.[1163]

Not all mAbs prove equally effective at neutralizing SARS-CoV-2, especially Omicron. For example, in one study recombinant mAbs of three Ig isotypes (IgG, IgA, and IgM), sharing the same anti-spike protein Fab region, were developed. When these were compared, the neutralization ability was dramatically higher for IgM and IgA than IgG mAbs.[1164] Fortunately, mAbs have held up well against variants, including some against Omicron.[1165]

[1159] Fischer et al., "The Role of Passive Immunization," 2–3.
[1160] Focosi, Tuccori, and Franchini, "The Road Towards Polyclonal Anti-SARS-CoV-2," 6.
[1161] FDA, "Convalescent Plasma EUA Fact Sheet," 1.
[1162] Martinez et al., "A Broadly Cross-Reactive Antibody," 2.
[1163] Dejnirattisai et al., "The Antigenic Anatomy of SARS-CoV-2 Receptor Binding Domain."
[1164] Pisil et al., "Is SARS-CoV-2 Neutralized More Effectively by IgM and IgA than IgG Having the Same Fab Region?" 2, specifies, "IgM and IgA neutralized the viral particles at 225-fold and 125-fold lower concentrations, respectively."
[1165] Takashita et al., "Efficacy of Antibodies and Antiviral Drugs against Covid-19."

At the end of April, 2022, the National Institutes of Health (NIH) offered its treatment guidelines for COVID-19 with respect to mAbs. It noted about emergency use authorization (EAU):

> Five anti-SARS-CoV-2 mAb products have received EUAs from the Food and Drug Administration (FDA). Bamlanivimab plus etesevimab, bebtelovimab, casirivimab plus imdevimab, and sotrovimab received EUAs for the treatment of mild to moderate COVID-19 in nonhospitalized patients with laboratory-confirmed SARS-CoV-2 infection who are at high risk for progressing to severe disease or hospitalization. The FDA issued an EUA for tixagevimab plus cilgavimab, a long-acting anti-SARS-CoV-2 mAb combination, as SARS-CoV-2 PrEP for individuals who do not have SARS-CoV-2 infection, who have not been recently exposed to an individual with SARS-CoV-2 infection, **AND** who are at risk for inadequate immune response to COVID-19 vaccination **OR** have a documented history of severe adverse reactions to a COVID-19 vaccine or any of its components.

NIH cautioned that the effectiveness of mAb treatments varies dramatically depending on the circulating variant. Among other things, they also recommended that treatment with anti-SARS-CoV-2 mAbs should be started as soon as possible after SARS-CoV-2 infection is confirmed by an antigen test or a nucleic acid amplification test and within 7 days of symptom onset.[1166]

Polyclonal antibodies ('hyperimmune serum') provide IgG formulations. These can be delivered intravenously, intramuscularly, or sometimes intranasally. By the end of 2020, some 16 clinical trials were underway, some using serum from convalescent patients and others using serum from immunized animals (e.g., horses and rabbits). A number of major plasma manufacturers united in early 2020 under the CoVIg19 Plasma Alliance to develop, test, and provide hyperimmune serum.[1167]

In August, 2020, the NIH through the National Institute of Allergy and Infectious Diseases (NIAID) initiated testing of a fully human polyclonal antibody (SAB-185) for treatment of COVID-19. The testing, in a program dubbed ACTIV-2, continued into phase III trials in early 2022.[1168] In March, 2022, enrollment in the trial was halted when the independent Data and Safety Monitoring Board (DSMB) advised terminating the trial due to "operational futility." Although the trial was then halted SAB Biotherapeutics continued to study trial results.[1169]

Monoclonal and polyclonal antibodies, as the names suggest, vary in their clonage. *Mono*clonal antibodies all descend from a common parent; *poly*clonal descend from several different immune cells. Thus the latter are more versatile in the epitopes they can utilize. In the following illustration we can easily visual differences between monoclonal and polyclonal antibodies.

[1166] NIH, "Anti-SARS-CoV-2 Monoclonal Antibodies" (Update of Apr. 29, 2022).
[1167] Focosi, Tuccori, and Franchini, "The Road Towards Polyclonal Anti-SARS-CoV-2."
[1168] NIH, "Large NIH Clinical Trial Will Test Polyclonal Antibody."
[1169] SAB Biotherapeutics, "SAB Biotherapeutics Reports NIH Is Discontinuing Phase 3."

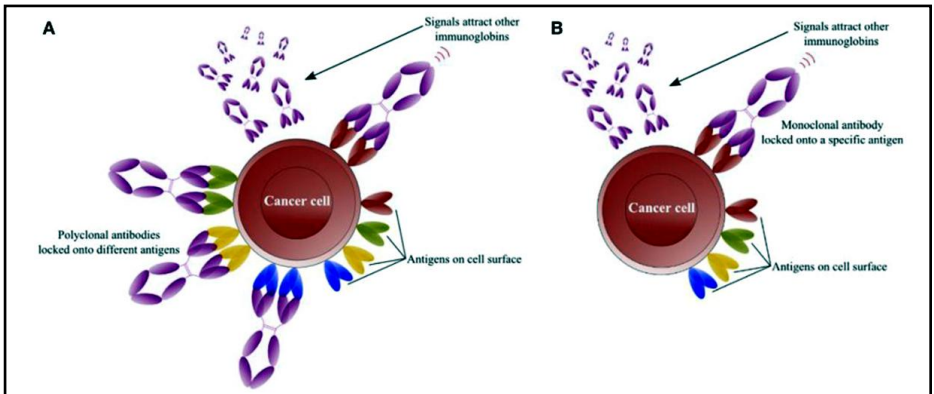

FIGURE 1 | Interaction of antibodies with numerous antigens present on the surface of target cell. **(A)** Interaction of polyclonal antibodies with specific surface antigen activates B lymphocytes to divide and differentiate into plasma cell clones producing more antibodies that recognize antigens. **(B)** Interaction of monoclonal antibodies with specific surface antigen activates B lymphocytes to divide and differentiate into plasma cell clones that further recruit homogeneous and mono-specific antibodies.

Illustration is from Abdullah F. U. H. Saeed, Rongzhi Wang, Sumei Ling, and Shihua Wang. "Antibody Engineering for Pursuing a Healthier Future." *Frontiers in Microbiology* 8 (Mar., 2017) art. 495. doi: 10.3389/fmicb.2019.00495. The material is from page 3 and is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/legalcode). The original has been changed in font size and type for explanatory material.

We also may note ways in which antibody treatments can be compared.

Table 2. A comparison of neutralizing antibody (nAb)-based therapies for COVID-19.

	CCP	Hyperimmune Serum (Polyclonal IgG)	Monoclonal Antibodies
Speed of access	Weeks (as soon as convalescents appear)	>1 year	>1 year
Safety issues	Safe (pathogen inactivation, possible plasma protein allergies)	Safe (solvent/detergent, but ABO-incompatible)	Extremely safe (recombinant technology)
Potency	Very high (high PRNT titer; includes neutralizing IgA and IgM, and factors other than antibodies)	High (no IgA; less IgG_3)	High (nanomolar IC_{50}) Very high for Ab cocktails

Illustration excerpted from Daniele Focosi, Marco Tuccori, and Massimo Franchini. "The Road towards Polyclonal Anti-SARS-CoV-2 Immunglobulins (Hyperimmune Serum) for Passive Immunization in COVID-19." *Life* 11 (2021) 144. https://doi.org/10.3390/life11020144. The material is from p. 6 and made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/legalcode). The original has been changed by deleting the bottom portion of the Table, which covers cost, logistics, and stability.

Vaccination does not *provide* antibodies but *provokes* the body to produce them. The manner in which this can be done is treated in the next chapter.

Anti-Inflammatories

Anti-inflammatory drugs aim to reduce the inflammation caused by COVID-19. A variety of antiviral drugs also show anti-inflammatory properties but here we focus on drugs other than those previously discussed. Consideration of such drugs relates to earlier consideration of the *inflammasome*—an 'inflammatory' (*inflanna-*) 'body' (*-some*) that is a biological complex that senses danger and initiates an inflammatory response. The following illustration should help reorient us, especially since it makes plain the central role of *Caspase-1*.

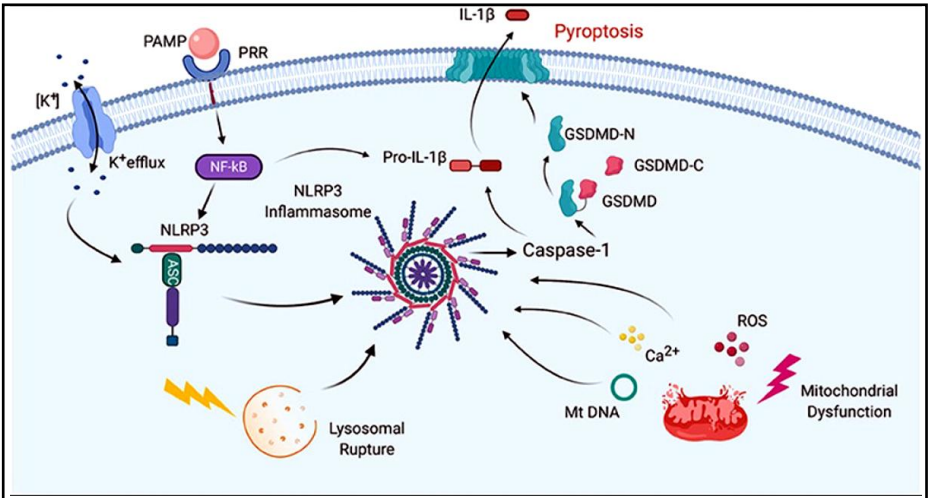

FIGURE 2 | Mechanisms of inflammasome activation. Inflammasome activation in general relies on two signals for its activation. First, a PAMP binds to a PRR resulting in synthesis of NLRP3 and pro-IL-1β. Then a second signal leads to inflammasome assembly, leading to the activation of caspase-1, processing of pro-IL-1β into IL-1β and pyroptosis. The second signal may come from a variety of pathways including K+ efflux. Lysosomal rupture or mitochondrial dysfunction. Mitochondrial dysfunction results in release of ROS, Ca2+ and mitochondrial DNA (mtDNA), all of which have been shown to activate the inflammasome. Pyroptosis occurs as a result of caspase-1-mediated cleavage of GSDM-D at the linker region of GSDM-D. Following GSDM-D cleavage, the amino terminus of GSDM-D (GSDM-D-N) forms a non-selective pore at the cell membrane through which IL-1β is then released.

Illustration is from Juan Carlos de Rivero Vaccari, W. Dalton Dietrich, Robert W. Keane, and Juan Pablo de Rivero Vaccari. "The Inflammasome in Times of COVID-19." *Frontiers in Immunology* 11 (Oct., 2020) art. 583373. https://doi.org/10.3389/fimmu. 2020.583373. The material is from page 3 and is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/ legalcode). The original has been changed in font size and type for explanatory material. Also see reproduced figure in Caruso et al., "Mechanism of Caspase-1 Inhibition by Four Anti-inflammatory Drugs Used in COVID-19 Treatment."

Some research has suggested that the key to anti-inflammatory drug treatment of COVID-19 is inhibition of caspase-1 (see preceding illustration), an enzyme involved in triggering the release of pro-inflammatory cytokines. Four anti-inflammatory drugs—colchine (see below), raloxifene (a selective estrogen receptor modulator (SERM)), dexamethasone and methylprednisolone (both corticosteroids)—were examined experimentally and all demonstrated caspase-1 site activity affinity. Since all four drugs also show activity against inflammation in COVID-19 the research helps explain why targeting caspase-1 may be therapeutically beneficial.[1170]

Non-steroidal anti-inflammatory drugs (NSAIDs), like ibuprofen, are ubiquitous in modern American over-the-counter treatment of pain and inflammation. There had been some theoretical concern that their means of operation, which includes increasing ('upregulating') availability of the ACE2 receptors that SARS-CoV-2 uses to attach and gain entry into host cells. This concern that NSAIDs might worsen cases of COVID-19 generated numerous studies. These did not find any increased risk of poorer COVID-19 outcomes.[1171] In fact, at least one report recommended early use of NSAIDs might prevent disease progression and even reverse lymphocyte depletion (*lymphocytopenia*).[1172]

One anti-inflammatory drug, *colchicine*, especially caught attention in late 2021. It is a drug known since the period of classical Greece, where it was employed against gout. In addition to anti-inflammatory effects, it acts as an immunomodulatory agent. With respect to SARS-CoV-2, it has been thought potentially useful for its ability to inhibit IL-1 production.[1173] By the end of 2021, a number of studies had researched the drug's efficacy against COVID-19, with mixed results. The most optimistic appraisal has been that taken at .6mg once a day the mortality rate might be cut in half. Other studies, however, found no benefit. The matter remains under investigation.[1174]

An innovative application of nanotechnology also has been proposed to combat inflammation in COVID-19. In one study highly biocompatible *glycyrrhizic acid* (GA) *nanoparticles* (GANPs) were synthesized based on GA, a natural plant ingredient which was already known for its anti-coronavirus activity. The GANPs, tested against the murine coronavirus MHV-A59, reduced the viral load in a manner suggestive of direct virus inactivation. Laboratory testing showed GANPs exhibit anti-inflammatory properties, too. Of special interest with respect to COVID-19 is that GANPs specifically target areas of inflammation, such as the lungs.[1175]

[1170] Caruso et al., "Mechanism of Caspase-1 Inhibition by Four Anti-inflammatory Drugs."
[1171] Kragholm, Torp-Pedersen, and Fosbol, "Non-Steroidal Anti-Inflammatory Drug Use."
[1172] Kelleni, "Early Use of Non-Steroidal Anti-Inflammatory Drugs in COVID-19," 2.
[1173] Soy et al., "Cytokine Storm in COVID-19," 2092.
[1174] Morrison, "Ancient Greek Drug."
[1175] Zhao et al., "Glycyrrhizic Acid Nanoparticles." Also see Sun et al., "Glycyrrhizic Acid: A Natural Plant Ingredient."

Antivirals

Antiviral drugs have caught considerable attention. The National Institutes of Health (NIH) summarize data on 8 separate antivirals or antiviral groups. The following Table offers an overview from the NIH information. [1176]

ANTIVIRAL	How They Work
Chloroquine or Hydroxychloroquine and/or Azithromycin	*Chloroquine* (CQ) and *Hydroxychloroquine* (HCQ) inhibit fusion of virus with host cell membrane and may block transport of virus for release; also have immunomodulatory effects. *Azithromycin* has both antiviral and anti-inflammatory properties. Used with HCQ has a synergistic effect.
Interferons (IFs)	Family of cytokines; those studied for treatment of COVID-19 include *interferon alfa* (based on IF-α), *interferon beta 1a* (IF-β1a), and *interferon lambda* (IFN- λ). Interferons 'interfere' with viral replication.
Ivermectin	FDA approved as an anti-parasitic drug, but not approved for COVID-19. *In vitro* studies suggest it inhibits the intracellular transport of the virus, its replication, and may interfere with viral S protein attachment to host cell membrane. But such effects would require doses *100x higher* than approved for use.
Molnupiravir*	Oral prodrug of *beta-D-N4-hydroxycytidine* (NHC), a ribonucleoside that has broad antiviral activity against RNA viruses by inducing lethal changes in viral RNA.
Nitaoxanide	A broad-spectrum thiazolide antiparasitic agent FDA approved to treat some conditions, but not COVID-19. It appears to interfere with viral protein processing at a critical point. It also inhibits pro-inflammatory cytokines.
Paxlovid*	Paxlovid is *ritonavir-boosted nirmatrelvir*. Both are protease inhibitors; ritonavir increases nirmatrelvir concentrations to a therapeutic level. It acts against viral replication.
Protease Inhibitors (Lopinavir/Ritonavir and other HIV proteases)	Protease inhibitors interfere with viral replication (specifically the cleavage of polyproteins into an RNA-dependent RNA polymerase and a helicase).
Remdesivir	Only FDA approved antiviral. A nucleotide prodrug of an adenosine analog, it inhibits viral replication.

*EUA = Emergency Use Authorization by FDA.

[1176] NIH, "Antiviral Therapy" (information as of May 26, 2022). Emphasis in original.

Prominent among antivirals in the first year or so of the pandemic were hydroxychloroquine and remdesivir. A multicenter study of four antiviral drugs (hydroxychloroquine, interferon beta 1a (IFN-β-1a, lopinavir, and remdesivir) repurposed to fight COVID-19 found no demonstrable effect on such vital factors as length of hospitalization or mortality.[1177]

Some studies have found both therapeutic efficacy and safety of using IFN-β-1a, either alone or together with other agents (e.g., hydroxychloroquine, lopinavir plus ritonavir). However, conflicting results from various studies have meant caution in drawing conclusions about the efficacy of these various agents.[1178] In general, interferon treatments have not been demonstrated efficacious and are typically discouraged.

In late 2021, Pfizer's antiviral drug *Paxlovid* (*nirmatrelvir [PF-07321332]*)—which actually combines two antivirals (nirmatrelvir and ritonavir)—was approved by the U.S. Food and Drug Administration (FDA). In December of that year the company announced results of its Phase 2/3 ***E****valuation of* ***P****rotease* ***I****nhibition for* ***C****OVID-19 in* ***H****igh-****R****isk Patients* (EPIC-HR) trial, which involved 2,246 adults. Compared against placebo, Paxlovid reduced the risk of hospitalization or death by 89% among high-risk adult patients if taken within three days of symptom onset (and 88% risk reduction if taken within five days of symptom onset).[1179] The drug uses a five day course of treatment.

However, in the first four months of 2022, anecdotal reports surfaced of 'rebound patients'—those who, having successfully completed treatment and were free of symptoms, subsequently experienced symptoms of COVID-19. Such experiences raised questions as to whether the drug was actually stopping the disease or merely suppressing symptoms. Or, if the drug was working perhaps in some people absorption was less, leaving enough viral material to reactivate COVID-19 after treatment ended. This idea logically raised the suggestion of an increased course of treatment, but under existing FDA guidelines at the time this was not a feasible option.[1180] More bad news came when Pfizer announced on April 29th that a large study trial indicated it was not effective at preventing infection by SARS-CoV-2 in individuals living with someone infected with the virus and taking the treatment for it.[1181]

Concerns were also raised about the efficacy of antivirals such as remdesivir and molnupiravir against Omicron because the variant has mutations in viral regions targeted by these drugs. However, a study of these two antivirals, as well as oral antiviral *Phosphate Prodrug of* PF-*00835231* (PF-07304814), found that all three might show efficacy against Omicron.[1182] Another report noted that anti-

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[1177] Murgolo et al., "SARS-CoV-2 Tropism," 2.

[1178] Sosa et al., "Effects of Interferon Beta in COVID-19." Cf. Wilkinson, "Subcutaneous Interferon Beta-1a in COVID-19."

[1179] Pfizer. "Pfizer Announces Additional Phase 2/3 Study Results."

[1180] White, "COVID-19 Is Recurring in Patients Who Took Pfizer's Antiviral."

[1181] Reuters, "Pfizer Says COVID-19 Drug Paxlovid Fails to Prevent Infection."

[1182] Takashita et al., "Efficacy of Antibodies and Antiviral Drugs against Covid-19," 997.

virals emdesivir, molnupiravir, and nirmatrelvir all appeared to be as effective against the highly infectious Omicron subvariant BA.2 as they are against earlier SARS-CoV-2 variants.[1183]

Cannabidiol

In early 2022, a peer-reviewed scientific article reported that high quality *cannabidiol* (CBD)—that with a purity of 97% or better—inhibits SARS-CoV-2 replication in human epithelial cells. CBD is a natural substance produced by the plant *Cannabis sativa*. The researchers found that CBD's inhibition of viral replication could occur under non-toxic conditions and that it was effective doing so against Wild Type (the original strain), Alpha, Beta, and Gamma variants. When metabolized in intestine and liver CBD's metabolite 7-hydroxy-cannabidiol (7-OH-CBD) also exhibited this inhibitory ability. The researchers note that analysis of host cells after viral infection and treatment with CBD reveals almost complete reversal of viral changes, but not a return to normal state; instead, the treated cells resemble those having experienced CBD alone (i.e., without virus). They suggest CBD may act to prevent viral protein translation. It might also be the case that CBD counters the virus' suppression of the interferon signaling pathway—an important part of immune system response. At the same time, it seems to dampen the inflammatory response that follows the virus provoking cytokine production. Finally, they point out that people with a medication record of CBD100 (i.e., CBD in oral solution of 100mg/mL) show a lower rate of infection than matched controls without such a medication record (6.2% compared to 8.9%).[1184]

Inhibitors

Inhibitors, as the name means, 'inhibit'—they aim, ideally, to prevent something, or at least to hinder it or in some manner restrain it. Drugs intended to inhibit SARS-CoV-2 typically target attachment and entry of the virus. For example, we saw that enzyme named transmembrane serine protease 2 (TMPRSS2), which is abundantly expressed in lung tissue and elsewhere, has an important role in the principal way the virus enters host cells. *TMPRSS2 inhibitors* (e.g., camostat and nafamostat), especially in combination, might be one way to stop infection. Similarly, we saw that the virus uses cathepsin-mediated endocytosis to enter host cells. *Cathepsin inhibitors* might interfere with the virus using this pathway. So, too, might other agents involved, such as those that interfere with the acidification process in endosomes that we discussed, or *inhibitors of endosomal maturation* (e.g. apilimod).[1185]

Among goals of research scientists is the development of effective antiviral medications to treat COVID-19. In this respect, aspects of viral replication are at the forefront. For example the main protease (M^{pro}) residing in nsp5, and the

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[1183] Takashita et al., "Efficacy of Antivirals against the SARS-CoV-2 Omicron Subvariant BA.2."
[1184] Nguyen et al., "Cannabidiol Inhibits SARS-CoV-2 Replication."
[1185] All of these are mentioned in Murgolo, "SARS-CoV-2 Tropism," 4.

viral replication-transcription complex (RTC), are thought to be promising targets for antivirals against SARS-CoV-2.[1186] Also RNA-dependent RNA polymerases (RdRps) are considered a major target for antiviral compounds.[1187]

Naturopathic and Homeopathic Treatment Alternatives

Science has long recognized the value of natural products with antiviral properties. In this regard, it has been pointed out, the pharmaceutical industry often turns to natural products with antiviral activity. In fact, with the SARS (SARS-CoV-1) outbreak at the beginning of the 21st century, a variety of natural sources from flora and fauna were tested for potential treatment use. These included natural metabolites in a number of chemical classes (flavones, flavonols, fatty acids, tannins, terpenes, and alkaloids).[1188]

The Table below offers an overview of some common natural products considered as possible help against SARS-CoV-2 and COVID-19.[1189]

Common Name	Scientific Name	Why They May Be of Value
Basil	*Ocimum basilicum*	3CLpro inhibitor (3CLpro is an enzyme of SARS-CoV-2)
Chamomile	*Matricaria recutita*	3CLpro inhibitor
Cinnamon	*Cinnamomum zeylanicum* Blume, or *Cinnamomum verum* J. Presl.	ACE2 inhibitor (ACE2 is the human receptor SARS-CoV-2 favors for attachment and entry) *and* 3CLpro inhibitor
Ginger	*Zingiber officinale*	3CLpro inhibitor
Grapes	*Vitis vinifera* L.	ACE2 inhibitor
Green tea	*Camellia sinensis*	TMPRSS2 inhibitor (*transmembrane serine protease 2* is an enzyme that primes and activates fusion of the virus with the host cell membrane)
Mushrooms	(of *Paxillus* genus)	TMPRSS2 inhibitor
Olives	*Olea europaea* L.	ACE2 inhibitor
Pepper	*Capsicum* spp.	ACE2 inhibitor

[1186] V'koski et al., "Coronavirus Biology and Replication," 162.
[1187] Mesters, Tan, and Hilgenfeld, "Viral Enzymes," 778.
[1188] Da Silva Antonio, Wiedermann, and Veiga-Junior, "Natural Products' Role against COVID-19," 23386.
[1189] Table based on Da Silva Antonio, Wiedermann, and Veiga-Junior, "Natural Products' Role against COVID-19."

Let us now look a bit more at a few natural products.

Herbs and Spices

Observing that spices regulate expression of different cytokines, some researchers have pointed to their possible efficacy in combating the inflammatory response seen in COVID-19. For example, cinnamon (and the essential oil extracted from it), in addition to possessing antiviral and immunomodulatory qualities, is anti-inflammatory. So, too, are ginger, mint, mustard, nutmeg extracts, and other natural materials. Some spices already have been used in clinical trials to combat cytokine storm—a condition in which the body's natural inflammatory response goes awry. For example, one of the compounds of the aforementioned cinnamon—β-caryophyllene at 126 mg daily for 8 weeks—was found, in a randomized double-blind placebo-controlled study, to have significant anti-inflammatory properties. It should be cautioned that these have *not* been shown to be efficacious in treating inflammation related to COVID-19. But a clinical trial using curcumin extract and piperine (an ingredient in black pepper) with COVID-19 patients had been initiated at the time of the researchers' report.[1190]

Traditional Chinese Medicines (TCMs)

The COVID-19 pandemic originated in China, a land were traditional medicine is still actively practiced, often alongside modern and Western medicine. Chinese national guidance early in the pandemic recommended as supplemental treatment the use of *Shufeng jiedu capsules* (SFJDC) and *Jinhua qinggan granules*. The capsules are a patented herbal drug composed of eight medicinal plants (*Polygonum cuspidatum, Forsythia suspensa, Isatis indigotica, Bupleurum chinense, Patrinia scabiosifolia, Verbena officinalis, Phragmites communis* and *Glycyrrhiza uralensis*). SFDJC targets a signaling pathway (*extracellular signal-related kinase* (ERK)) important in modulating anti-inflammatory and immunomodulation activity. SFDJC has been found in studies to decrease anti-inflammatory factors IL-6, IL-10, TNF-α, and IFN-γ. A clinical study found that when combined with conventional antiviral drug treatment there was a significant reduction in the symptomatic phase of COVID-19. Jinhua qinggan granules—described as brown, with a slightly fragrant smell but bitter taste—are comprised of a dozen traditional Chinese medicine ingredients (honeysuckle, gypsum, honey ephedra, fried bitter almond, Scutellaria, Forsythia, Fritillaria, Anemarrhena, burdock seed, Artemisia annua, peppermint, and licorice.). They are reputed to be able to significantly inhibit SARS-CoV-2 replication. These granules also target signaling pathways (TNF, MAPK, and T-cell receptor ones). A study has found them useful in relieving fever, fatigue, poor appetite, and diarrhea.[1191]

[1190] Kunnumakkara et al., "COVID-19, Cytokines, Inflammation, and Spices," 9–16. With respect to the clinical trial, see Miryan et al., "Effects of Curcumin-Piperine."

[1191] Nile and Guy, "Recent Clinical Trials," 11–12; Xia et al., "Shufeng Jiedu;" An et al., "Efficacy of Jinhua Qinggan Granules."

Immunity

In the fall of 2021, after reviewing and analyzing a broad range of studies, the Centers for Disease Control and Prevention (CDC) concluded, "Available evidence shows that fully vaccinated individuals and those previously infected with SARS-CoV-2 each have a low risk of subsequent infection for at least six months." However, the same brief immediately noted: "The immunity provided by vaccine and prior infection are both high but not 100%."[1192] Put more succinctly, whether by virtue of infection or vaccination enough immunity generally results to feel protected for at least half a year (maybe).

Before we can assess how good this news might be, we need to understand what 'immunity' is. In doing so we also need to consider two kinds:

- individual immunity; and
- herd immunity.

Individual Immunity

'Immunity' is a word defined in various ways by different groups. In the wider public the general sense embraced by most people seems to follow the derivation of the word from Latin (*immunis*), where it means 'exempt.' Usually folk think of immunity from disease as exemption from it—like a Get-Out-of-Jail-Free card that never has to be played. Unfortunately, that is a misinformed understanding. It is a myth that either vaccination or infection with COVID-19 guarantees immunity in the sense of 'No more worries folks! I'm completely protected forever!'

A better understanding of the term comes from biology, where it refers to the body's ability to *recognize* what properly belongs and what doesn't, and then *respond* appropriately—hence, the idea of an 'immune system'. Or we might turn to medicine where the basic sense is *protection* (relative), and many different permutations of immunity are recognized. For our purposes, which aim to understand immunity in terms of disease, *immunity is protection mediated by resistance*. Both the ideas of 'protection' and 'resistance' matter in comprehending immunity. The quality of resistance is highly variable, and only a few fortunate individuals have an immune profile characterized by high production of cytokines upon stimulation by pathogens that has been correlated with early clearance of an infecting pathogen and with so-called 'natural resistance' to infection.[1193] Most of us do not have the kind of natural resistance that short-circuits infections so quickly we may not know we even had them.

So, immunity does confer protection against COVID-19 but it does so as a degree of resistance that is influenced by various factors. That means *immunity is not the same for everyone*. For most people, *immunity is limited protection*.

[1192] CDC, "Science Brief: SARS-CoV-2 Infection-Induced and Vaccine-Induced Immunity."
[1193] Netea et al., "Natural Resistance Against Infections," 112.

Herd Immunity

'Herd immunity' is a concept that takes the basic ideas of immunity as applied to individuals and applies them instead to large populations. Thus, *herd immunity is the protection provided by communal resistance*. The immunity depends on the herd. If enough people mount effective resistance then the virus cannot make much headway. The fewer the number of people who buy into active resistance (e.g., through vaccination) the less likely herd immunity is. With any given disease it is an open question how many people have to mount effective resistance for the whole population to be reasonably well protected.

The fact is, even if we hypothesize that herd immunity to the COVID-19 virus is achieved with 50% population immunity, we don't know how long such immunity lasts. Researchers Arnaud Fontanet and Simon Cauchemez point out that immunity to seasonal coronaviruses is usually short-lived, and this may be even more the case for those who contract mild cases of infection. Thus it might take repeated cases of re-infection to achieve the kind of strong protection everyone desires.[1194] Moreover, as one recent study warns, the rapid viral adaptations shown by the COVID-19 virus (SARS-CoV-2) mean it is possible herd immunity will *never* be achieved.[1195]

The bottom line for both individual immunity and herd immunity is much the same: the protection provided depends on effective resistance and is variable. *No one has any guarantee of sustained immunity*.

Breakthrough Infections

Even those vaccinated can experience infection by SARS-CoV-2 (just like those receiving the annual flu shot might still get the flu). When such happens it is termed a *breakthrough infection*, because the virus has 'broken through' the layer of protection afforded by the vaccine. But just as armor can deflect a sword strike, the vaccine blunts the killing power of the virus, significantly lowering the risk of severe illness and/or death. Given the reality of breakthrough infections some argue that vaccination is pointless. But research persuasively demonstrates otherwise.

A study reported in mid-December, 2021, found dramatic benefits from vaccination in those who subsequently became infected with SARS-CoV-2. Researchers in Oregon examined 26 persons (77% female) fully vaccinated with the Pfizer vaccine. Of these 21 (81%) reported mild symptoms with the breakthrough infection. Despite mild disease adaptive immunity showed substantial boosting. Not only was there a solid increase in antibodies produced, but these appeared to be significantly effective neutralizing the virus.[1196] Corresponding author for the study, Fikadu G. Tafesse, observed in a news release, "You can't get a better immune response than this. Our study suggests that individuals who

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[1194] Fontanet and Caucheme, "COVID-19 Herd Immunity."
[1195] Dopico, et al., "Immunity," 12.
[1196] Bates et al., "Antibody Response and Variant Cross-Naturalization."

are vaccinated and then exposed to a breakthrough infection have super immunity."[1197] (We shall look at this further under the notion of hybrid immunity.)

Reinfection

Viral reinfection depends in part on the virus involved. In those that cause systemic infection (e.g., hepatitis A, measles, mumps, polio, and rubella) with presence of the virus in blood (*viremia*), reinfection is very uncommon. In viruses that cause mucosal infections without viremia (e.g., influenza and seasonal human coronaviruses), reinfection is common. In fact, with endemic human coronaviruses—the so-called 'seasonal' ones—the mean time to reinfection ranges from 30–55 months, depending on virus.[1198] Given the increasing similarity of SARS-CoV-2 to these less dangerous human coronaviruses, we can reasonably expect to find reinfections.

Real-world evidence shows that reinfection by SARS-CoV-2, with subsequent COVID-19, does occur. Especially with variants prior to Omicron, many large scale studies found it to be a rather insignificant factor by being less than 1%.[1199] Research published early in 2022 of reinfections in Madrid, Spain, found that of 41,195 cases of documented infection between March, 2020–March, 2022, just 93 reinfections (0.23%) were uncovered. An even smaller percentage was from reinfection by the same strain of the virus.[1200] However, despite the apparent rarity, reinfections—even by the same strain—have been documented. Individual experience can vary and other factors may convene to make reinfection more likely in some environments.

The disease, unfortunately, is not a 'one-and-done' experience for many people. For example, a nationwide study in Denmark reported 0.65% of those with a virus-positive confirmed infection in the first wave of the pandemic also tested positive in the second wave.[1201] It also remains an open question whether reinfection is more likely to be associated with worse symptomatology or a better condition. The Madrid study mentioned above found the second infection was generally worse.[1202] More importantly, Omicron has challenged any rosy outlook.

Omicron and Reinfection

The extreme infectiousness of Omicron seemed in early studies to return rates of reinfection of 9.5% or higher.[1203] Omicron proved more likely to cause reinfection than the Delta variant had. An Imperial College COVID-19 Response Team in England, in a mid-December, 2021 report, said, "Controlling for vaccine status, age, sex, ethnicity, asymptomatic status, region and specimen date,

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[1197] Taffese quoted in Robinson, "Breakthrough Infections Generate 'Super Immunity'."
[1198] Cohen and Burbelo, "Reinfection with SARS-CoV-2," e4225.
[1199] Luo et al., "Infection Fatality Ratio and Case Fatality Ratio of COVID-19," 45.
[1200] Rodriguez-Grande et al., "Systematic Genomic and Clinical Analysis."
[1201] Rodriguez-Grande et al., "Systematic Genomic and Clinical Analysis," 91.
[1202] Rodriguez-Grande et al., "Systematic Genomic and Clinical Analysis," 92.
[1203] UK Health Security Agency, "SARS-CoV-2 Variants" (Technical Briefing 33), 4, 28.

Omicron was associated with a 5.41 (95% CI: 4.87-6.00) fold higher relative risk of reinfection compared with Delta." They specifically noted that immunity from natural infection provided no special protection against Omicron reinfection. [1204] By mid-May, 2022, many epidemiologists were raising the spectre of reinfections becoming more common because of Omicron's subvariants.[1205]

When the Omicron variant emerged in 2021 in the countries at the southern end of Africa, a study in South Africa of 211,000 COVID-19 patients found rates of reinfection increasing regardless of which previous variant a person had been infected by. Specifically, relative to those with prior infection:

- those infected by the original (Wild Type) virus strain had a 73% chance of reinfection with Omicron;
- those infected in the second wave (Beta variant) had a 60% reinfection rate with Omicron; and,
- those infected in the third wave (Delta variant) had a 40% reinfection rate with Omicron.[1206]

These findings raise an obvious question about the duration of immunity.

How Long Does Immunity Last?

In light of these facts it is natural to ask, How long does immunity last? Researchers studying the matter typically set *6 months* as a baseline period.

First, we need to note that certain underlying truths govern answering the question of how long immunity lasts. These include, among others:

- strength of the individual immune system;
- frequency and length of exposure to SARS-CoV-2; and,
- severity of any infection.

Note: the question of whether the immunity is via vaccine or infection is left aside here. The above factors apply to both scenarios—vaccinated or not.

If a person has a weak or compromised immune system, *then* it may take less exposure to become sick and when sick might become sicker than others. *If* a person has a strong immune system but experiences prolonged exposure, *then* they are likely to be infected, but whether that infection leads to severe illness—or any at all—depends on numerous other factors. *If* a severe infection results—a possibility no matter the strength of the immune system, *then* all bets are off.

We noted before that antibody levels peak within 3–5 weeks after infection, then begin to wane at variable rates among individuals. The fastest to decline seem to be binding antibodies against N protein, then those against the RBD of the spike (S) protein, and finally against S protein itself. Still, SARS-CoV-2 specific IgG antibodies remain detectable in about 90% of people who produce

[1204] Ferguson et al. (for the Imperial College COVID-19 Response Team), "Report 49," 7.
[1205] Mandavilli, "How Often Can You Be Infected with the Coronavirus?"
[1206] Discovery Health, "Discovery Health."

antibodies 10–12 months after infection. Neutralizing antibodies decline significantly within the first 2–3 months, but then decline slows.[1207]

Such information does not in itself tell us how long any given person will enjoy some immunity against infection by SARS-CoV-2 and subsequent COVID-19. Because it has been relatively easier to study vaccinated people than those infected, and because vaccination produces more level immunity effects across people than infection's 'natural' immunity does, scientists are more confident about how long protection persists after vaccination. In late 2021, the CDC reported it as about 8 months (250 days), with an initial 90% effectiveness rate declining over that period to about 70%. Among the unvaccinated who acquire immunity 'naturally' through infection, we noted above that some antibodies (notably IgG) remain detectable in most people for up to 10–12 months.[1208]

The bottom line, in one respect, is: 'We don't know how long immunity will last across people in general, let alone for any given individual.' A foolish reaction to this is to decide, therefore, whatever one believes about their individual risk is as valid as any other belief. Prudence dictates, instead, to suspend belief and prepare for the worst. Just as gravity doesn't care that someone believes they can fly when they step off a cliff, SARS-CoV-2 doesn't care if someone believes they won't get sick, or if they do it will just be mild.

Is 'Natural Immunity' Superior to Vaccine Immunity?

Resistance to vaccination uses a basic logic that seems to run like this:

- Most people won't be infected, so taking a vaccine is a risk.
- If a person is infected the chance of serious illness is quite small, and less than the risk from vaccine side-effects.
- For the generally small price of no symptoms or mild ones a person who has been infected is conferred the advantage of natural immunity without any of the risk of a vaccine.

A common anti-vaxxer claim is that *natural immunity*—protection acquired through infection by the virus—is superior to any conferred by a vaccine. Here we will set aside the first two points in the above argument and examine only the matter of whether 'natural immunity' is indeed preferable to vaccination.

Implicit in the idea that natural immunity is superior to immunity from vaccines are one or the other—or both—of these presumptions:

- natural immunity is better because it is 'natural' not artificial like a vaccine provides (presuming 'natural' is always superior to 'manufactured'); and/or
- natural immunity is better because it lasts longer.

[1207] CDC, "Science Brief: SARS-CoV-2 Infection-Induced and Vaccine-Induced Immunity."
[1208] CDC, "Science Brief: SARS-CoV-2 Infection-Induced and Vaccine-Induced Immunity."

We could say that the first point is an argument about *cause* while the second point is an argument about *effect*.

Anti-Vax Contention 1. Natural immunity is better because it is 'natural.'

The first idea draws on the informal logic that appeals to a common presumption among people that somehow anything natural is superior to anything man-made. Of course, put that way most people readily see how absurd that general idea is. Not many would want us to give up the machines and technology that make life so comfortable in favor of trying to recreate an Adam and Eve situation in the wilderness.

So the notion is often confined to just matters of health and medicine. Professional anti-vaxxers, who peddle their own collections of elixirs and 'natural' remedies, profit from people buying into this philosophy and thus buying their products. But again, a moment's thought brings qualifiers right and left. Not many are going to argue that we should give up glasses, hearing aids, or even band aids because they are artificial helps and because the 'natural' condition of not seeing well, hearing well, or having a wound is better.

So the logic is further restricted. What the proponents *really* have in mind, they'll say, is the natural body as God intended—healthy and free from defect. So while natural means such things as good nutrition, sensible eating habits, sound sleep hygiene, and reasonable exercise as a primary course of action, it is all right to supplement these as needed by drawing on medicine, especially those forms that use or derive their products directly from Nature itself.

But now the line seems arbitrarily drawn. Who decides how artificial something is before it is rejected? How are glasses, hearing aids, or band aids at all natural? Something like an mRNA vaccine, which copies Nature closely, is clearly more 'natural' than a band aid. So why does the latter get universal approval while the other is controversial? If the standard is something like 'restoring natural function' then glasses (restoring healthy sight) and vaccines (restoring healthy immune function) both seem to qualify.

However, some may object that the real comparison/contrast ought not to be glasses versus vaccines, but natural infection versus vaccines. Allowing Nature to take its course and having the immune system strengthened by infection is more 'natural' and preferable to the insult to the body of a vaccine shot. An immediate problem with this line of reasoning is that it already contravenes the premise that 'natural' means 'healthy and whole'—as God intended. Viral infection doesn't qualify. So how can infection be more 'natural' than vaccination, if neither is a signal of health and wholeness?

And if the focus is shifted to the health and wholeness of the immune system, recall the lessons from earlier in this chapter. As good as it is, the immune system is far from perfect. SARS-CoV-2 has developed effective ways to evade immune surveillance to establish itself, and even found ways to co-opt immune system cells so that they work for the virus rather than against it. Moreover, as those who study SARS-CoV-2 and treat COVID-19 are quick to remind us, who

gets infected, who gets sick, and how sick they get all remain at least a bit mysterious.[1209] The bottom line is that taking a chance on infection to acquire natural immunity is exactly that—taking a chance. It is a risky bet.

We might ask a second question about the immune system: how does infection make that system healthier or more whole than a vaccine? The immune system draws no distinction between foreigners—the virus and the vaccine are treated alike. Yet with the virus the immune system often suffers much worse, not only not becoming healthier as a result of the experience, but suffering long term damage and even complete collapse leading to death. By contrast the vaccine regularly strengthens the immune system with far less risk of negative consequences. So which really supports what is 'natural'?

Anti-Vax Contention 2: Natural immunity is better because it lasts longer.

The second argument—one from effect—is probably the one heard more often. We saw in the preceding section on immunity that protection tends to be limited. Neither immunity acquired through vaccination nor from infection guarantees long protection, let alone life-long protection. That is especially so the more infectious the SARS-CoV-2 variants become. For example, early data on Omicron—the most infectious variant to date—showed that reinfection by Omicron after previous infection by an earlier variant could easily occur within two to three months after the earlier infection.[1210]

Let's get more specific. We noted earlier that researchers set *6 months* as a baseline when measuring immunity. But one central tenet emerge from all the research: *No matter how long some measure of immunity remains, it declines over time.*

All studies demonstrate that fact, so let us suffice with a single example, using immunity acquired through infection. A study (April–August, 2020) of 156 frontline health care personnel (median age: 38; 60.3% female) at 13 hospitals tested them for antibodies to SARS-CoV-2 at two times, separated by about a 60 day interval. Of these people, 108 (69.2%) reported one or more symptoms of COVID-19, but without any hospitalizations. During the period of testing antibody levels, 93.6% of those who had showed detectable antibodies at the first test date displayed a decline in antibody response as measured at the second date. More than a quarter (28.2%) showed so much decline as to be below the level for positivity (i.e., to look as though they had never been infected).[1211]

Some folk are reluctant to acknowledge the limited duration of immunity, probably because it provokes a measure of anxiety. To repeat what was said before: the virus does not care what one chooses to believe. Granting that im-

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[1209] For a brief urvey of the matter, see Weintraub, "Why Do Some People Seem Unable to Catch COVID?"

[1210] Health Security Agency, "SARS-CoV-2 Variants" (Technical Briefing 33), 4. This early report, with data through December 20, 2021, less than 1 month after the time—Nov. 27—the first cases in England were reported, found 290 cases of reinfection within this 60-90 day time frame.

[1211] Self et al., "Decline in SARS-CoV-2 Antibodies after Mild Infection."

munity is limited in either situation, the question turns toward whether immunity conferred from infection—'natural immunity'—or that acquired through vaccination confers longer lasting immunity. It is that question that will occupy most of our attention through the remainder of this chapter in order to test the claim by anti-vaxxers that natural immunity is superior because it lasts longer.

Lessons from Research

Anti-vaxxers sometimes try to make use of scientific research for their own ends. Thus they are drawn to research which (at least by its title) appears to back up claims about the superiority of natural immunity. This often provides a veneer of scientific respectability even if void of any real scientific validity. Unfortunately, a scientifically illiterate population sees the appeal made to scientific titles by anti-vaxxers and concludes they must have solid scientific backing to their claims. Even if they take time to actually examine the scientific articles being pointed at they may not understand how anti-vax authors are misrepresenting the science reported in those articles. Let's look at a few examples.

Study 1

One study cited by anti-vaxxers as proof of their claims for life-long immunity for anyone who acquires natural immunity is titled "Immunological Memory to SARS-CoV-2 Assessed for Up to 8 Months after Infection." The title alone refutes any claim made that durable immunity lasts longer than eight months; the study was only assessing that period of time. The research is a study of B cells, $CD8^+$ T-cells, and $CD4^+$ T-cells, all of which play a role in 'immune memory,' and all of which operate in relative independence of one another. The study examined 188 people (108 female; 80 male) presenting with a range of asymptomatic, mild, moderate, and severe COVID-19 cases. The researchers explicitly remind us, "It is well recognized that the magnitude of the antibody response against SARS-CoV-2 is highly heterogeneous between individuals. We observed that heterogeneous initial antibody responses did not collapse into a homogeneous circulating antibody memory; rather, heterogeneity is also a central feature of immune memory to this virus." In other words, immune response to SARS-CoV-2 is *highly variable among people*. No universal claim can be made about how all people will have the same or similar antibody response, *or* immune memory. Further, the authors observe that both the severity of COVID-19 and factors like gender contribute to heterogeneity in immune memory. The bottom line, they say, is that "the source of much of the heterogeneity in immune memory to SARS-CoV-2 is unknown and worth further examination."[1212]

Study 2

A second study cited in support of the claim natural immunity may be life long,' is titled "SARS-CoV-2 Infection Induces Long-lived Bone Marrow Plasma Cells in Humans." Once more the title should at least tip one off that the

[1212] Dan et al., "Immunological Memory." The quotes are from pp. 8, 9, respectively.

article is making a rather limited and specific claim, not a global one. The authors early on, with the existing scientific literature in mind, state, "Reinfections by seasonal coronaviruses occur 6 to 12 months after the previous infection, indicating that protective immunity against these viruses may be short-lived. Early reports documenting rapidly declining antibody titres in the first few months after infection in individuals who had recovered from COVID-19 suggested that protective immunity against SARS-CoV-2 might be similarly transient." Their own study was both limited in scope—19 convalescent individuals who had experienced mild infection—and focused in targeting bone marrow plasma cells. Their interest in such cells stems from the role they play in supplying antibodies. They studied bone marrow samples ('aspirates') from the 19 participants and in 15 of them detected SARS-CoV-2 S-specific BMPCs (Bone Marrow Plasma Cells), indicating humoral immune memory at 7 months after infection. This is sufficient to be termed "long lived"—the term seized upon by anti-vaxxers—and indicates that alongside S-binding circulating memory cells the human immune system has a robust response to SARS-CoV-2.[1213] This is good news, but it is *not* the same as saying one thus acquires life-long immunity after a mild infection.

Study 3

A third study cited by anti-vaxxers, also in support of the claim of life-long immunity from infection, is titled "Comparing SARS-CoV-2 Natural Immunity to Vaccine-induced Immunity: Reinfections Versus Breakthrough Infections." This is a preprint article (i.e., before peer review) and compared three groups in Israel: one had received both doses of the Pfizer vaccine and had been uninfected (the reference group), another was unvaccinated and had been previously infected with SARS-CoV-2, and the third had both been previously infected and received a dose of vaccine. The study acknowledges that short-term effectiveness of the vaccine has been demonstrated; they were interested in its longer term effectiveness when compared to natural immunity via infection. They note their study was done at a time when the Delta variant predominated and that long-term effectiveness across variants is unknown. They note the long-term effectiveness of previous infection remains unknown, too—an important point anti-vaxxers like to ignore. Their research found a significant advantage in long-term protection via natural infection. They speculated this might be because the immune response is to actual SARS-CoV-2 spike proteins rather than to the artificial anti-spike protein immune activation spurred by the vaccine. But they emphasize this is just a hypothesis. After noting several limitations to their study they conclude that natural immunization does seem to confer "longer lasting and stronger protection" than vaccination, *but* they also report that vaccination after infection provides even better protection.[1214] Thus they are *not* supporting vaccine resistance. This study does provide support for an argument that natural

[1213] Turner et al., "SARS-CoV-2 Infection." The quote is from p. 421.
[1214] Gazit et al., "Comparing SARS-CoV-2 Natural Immunity to Vaccine-induced Immunity."

immunization may be stronger than vaccine immunization, but that is not the same thing as saying it is automatically life-long and that vaccination is not advantageous. The study says neither of those latter things.

Study 4

Another preprint article, released just before Thanksgiving, 2021, is titled "Continued Effectiveness of COVID-19 Vaccination among Urban Healthcare Workers during Delta Variant Predominance." This article reports a study of health care workers in urban Massachusetts during the period December 16, 2020 through September 30, 2021. The studied population was a diverse group (55% White, 20% Black, 13.5% Hispanic, 9% Asian). They found for fully-vaccinated health care workers (N=4,615) an adjusted vaccine effectiveness (VE) of 82.3%. A secondary study with a shorter time frame (July 1 to September 30, 2021), a period when the Delta variant of SARS-CoV-2 dominated, found an adjusted vaccine effectiveness of 76.5%. This group was then briefly compared to a smaller one (N=423) in which COVID-19 infection had occurred before vaccination. This group showed no reinfection prior to vaccination, and none after vaccination.[1215]

In both groups vaccination appears to be a plus (see again the article's title). Among the larger study group the incidence rate from before vaccination to after vaccination dropped from 5.2% to 0.6%. In the smaller group, which had experienced infection before vaccination, the incidence rate dropped from 100% to 0%. Obviously this is a dramatic drop, even though with a much smaller group and only over a relatively short period of time. While the authors believe this work contributes further evidence of natural acquired immunity, what it mostly does is confirm previous findings that indicate even after having survived an infection there is benefit to vaccination. It may well be that some natural immunity acquired before vaccination added benefit, but there is no way to definitively sort out how much of the 0% reinfection rate can be attributed to natural immunity or to the vaccine; the fact is that all that can be said is *both* may have been factors.

Study 5

Based on the accumulation of data into late 2021, the *Morbidity and Mortality Weekly Report* (Nov. 5, 2021) related that, "Among COVID-19–like illness hospitalizations among adults aged ≥18 years whose previous infection or vaccination occurred 90–179 days earlier, the adjusted odds of laboratory-confirmed COVID-19 among unvaccinated adults with previous SARS-CoV-2 infection were 5.49-fold higher than the odds among fully vaccinated recipients of an mRNA COVID-19 vaccine who had no previous documented infection (95% confidence interval = 2.75–10.99)." In simpler terms, the unvaccinated are at more than 5 times the risk for hospitalization even after acquiring so-called 'natural immunity' from previous infection 3–6 months earlier. The report con-

[1215] Lan et al., "Continued Effectiveness of COVID-19 Vaccination."

cluded, "vaccine-induced immunity was more protective than infection-induced immunity against laboratory-confirmed COVID-19."[1216]

Study 6

In early 2022, the CDC released a study entitled "COVID-19 Cases and Hospitalizations by COVID-19 Vaccination Status and Previous COVID-19 Diagnosis—California and New York, May–November, 2021." These two populous states accounted for about one-sixth of COVID-19 deaths in the U.S. through the end of November, 2021. This period coincided with the emergence and soon dominance of the Delta variant. The researchers noted a distinct shift between the risks of infection for adults (ages 18 and older) before and after the advent of Delta. Here's what they found (reporting first California (CA) and then New York (NY) hazard rates) for the lowered risk of COVID-19 when compared to the base group of unvaccinated people:

Before Delta base group ↓			(age-adjusted hazard rates) ←
Unvaccinated (no previous confirmed COVID-19 diagnosis)	**Unvaccinated** (with previous confirmed COVID-19 diagnosis) *'natural immunity'*	**Vaccinated** (no previous confirmed COVID-19 diagnosis)	**Vaccinated** (with previous confirmed COVID-19 diagnosis)
--	9.6-fold lower (CA) 8.5-fold lower (NY)	**19.9-fold lower** (CA) 18.4-fold lower (NY)	7.2-fold lower (CA) 9.9-fold lower (NY)
After Delta (by Oct. 3) --	29-fold lower (CA) 14.7-fold lower (NY)	6.2-fold lower (CA) 4.5-fold lower (NY)	**32.5-fold lower** (CA) 19.8-fold lower (NY)

The most pronounced lower risk rate *before* Delta was among the vaccinated; *after* Delta gained ascendancy the lowest risk was among the vaccinated who had also experienced infection by SARS-CoV-2. The authors draw attention to the effect of Delta, plus other factors such as the natural and expected decline of vaccine effectiveness over time.[1217]

If all one looks at is the difference after Delta became dominant between the risk for the previously infected unvaccinated compared to the vaccinated a very misleading conclusion can be drawn. At least one reporter headlined his account of the study as "CDC Says Natural Immunity Outperformed Vaccines Against Delta Strain."[1218] That may make for an attention-getting headline, but it is *not* 'what the CDC said.' In fact, right on page 1 of the report the authors write, "These results demonstrate that vaccination protects against COVID-19

[1216] Bozio, "Laboratory-Confirmed COVID-19."
[1217] León et al., "COVID-19 Cases and Hospitalizations."
[1218] Houseman, "CDC Says."

and related hospitalization, and that surviving a previous infection protects against a reinfection and related hospitalization." Let's emphasize here the word *surviving*—the authors are not promoting going out and getting COVID-19 as a way of becoming immune. They go on to point out that the difference between the before and after changes in risk hazard for the vaccinated can be accounted for by two things—Delta variant was better at immune evasion, and protection from vaccination (or natural infection) wanes over time. The basic point remains the comparison against the unvaccinated who have not yet been infected.

This latter point is especially well made in a striking figure (p. 5) that they provide. The relative hazard risk for all three groups who have acquired some form of immunity is staggeringly lower than for the unvaccinated who are not (yet) infected.

FIGURE. Incident laboratory-confirmed COVID-19–associated hospitalizations among immunologic cohorts defined by vaccination and previous diagnosis histories — California, May 30–November 13, 2021[*,†]

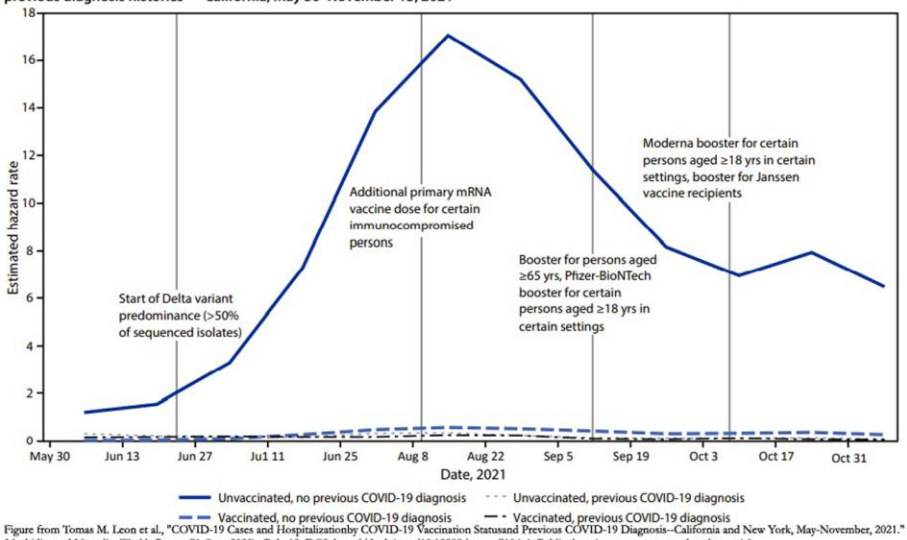

Figure from Tomas M. Leon et al., "COVID-19 Cases and Hospitalizationby COVID-19 Vaccination Statusand Previous COVID-19 Diagnosis--California and New York, May-November, 2021." *Morbidity and Mortality Weekly Report* 71 (Jan., 2022) ePub: 19. DOI: http://dx.doi.org/10.15585/mmwr7104e1. Public domain government-produced material.

Please note that no respectable scientist is making blanket statements about the superiority or inferiority of natural immunity compared to vaccination. All of the studies cited, and many more, point out that the matter is multifaceted. But they also agree that relying on natural immunity over choosing vaccination is a risk. As the authors above put it, natural immunity depends on *surviving* the infection. Millions of people have not.

Other Studies

The studies summarized above are just a few of the many that have examined the question of natural immunity versus that conferred by vaccination. Among scientists there is consensus that vaccination offers safe and effective protection absent the risks attending infection. The immunity conferred by vaccination is sufficient.

That, however, does not mean scientists ignore the evidence that suggests the natural immunity resulting from (surviving) infection may be stronger in at least some respects than that produced by vaccination. For example, a small study (39 COVID-19 patients compared to 11 vaccinated persons) found that in both situations significant induction occurred of natural killer cell-mediated *antibody-dependent cellular cytotoxicity* (ADCC)—an immune defense against SARS-CoV-2. The ADCC response in those who survived natural infection was stronger, suggesting it might provide better protection. However, the authors of the study also note that ADCC activity increases with disease severity.[1219] As we have seen elsewhere, this points at the possibility that the benefit from infection may depend on surviving more profound infection and more severe symptoms—a steep price to pay for the (possibly) better protection.

The bottom line in terms of research studies is that we are a long way from having any final, authoritative 'proof' that many anxious minds desire. Risking infection by opting not to vaccinate is a choice for risk far greater than any attending vaccination. But for those who take such a risk, and survive it, there may be some benefits from immunity resulting from infection. However, *the evidence to date suggests any superior benefit from immunity through infection is modest at best*. In the eyes of experts, the risk far exceeds the possible benefit.

Lessons from Logic

Often anti-vaccine arguments are superficially attractive because they appeal to commonly shared presumptions. They rely on informal logic—the shared sense that what *seems* true *is* true—and often lack what sound arguments must have—support. Here we can very briefly consider three arguments related to the question of immunity.

Argument 1

A tremendous advantage is provided by vaccination over relying on infection to achieve 'immunization.' In both cases a human body begins with *no* antibodies to the SARS-CoV-2 virus that causes COVID-19. The vaccinated body, though, pre-emptively begins making antibodies so that if and when the virus is en-countered, *the body is already prepared to fight*. The unvaccinated body must react on-the-fly, with no previous preparation. It is disadvantaged at the start.

Argument 2

With the COVID-19 virus it seems there are three alternatives:

- Avoid exposure;
- Accept exposure and infection in the name of achieving 'natural immunity'; or
- Accept vaccination as a different way to achieving immunity.

[1219] Rieke et al., "Natural Killer Cell-Mediated Abtibody-Dependent Cellular Cytotoxicity," 5.

It seems fair to guess most people like the first alternative best. But it has proven difficult to achieve. So, most people reckon that one or the other of the acceptances—of infection or vaccination—is probably inevitable. So which is preferable? We know from the grim statistics the price being paid for accepting the path of 'natural immunity' through infection. It is like playing Russian roulette; yes, there may be more empty chambers (i.e., a slim chance of serious illness or dying), but that is no comfort to the unlucky ones. And as the sheer volume of infections, serious illnesses, and deaths show, a lot of people are unlucky.

Argument 3

Let us, for the sake of argument, grant that the protection afforded by actual infection is superior to that conferred by vaccination. It may well be; various studies indicate an advantage. But that presupposes three things:

- *surviving* the infection in the first place;
- *avoiding the long-term effects and deficits* that often follow infection; and,
- having been *sick enough the first time* to acquire strong and lasting immunity.

All three conditions are important to consider if choosing the path of infection to achieve natural immunity. A study on herd immunity through a policy of allowing a large fraction of the populace to become infected (as advocated by anti-vaxxers) warns, "Unchecked, the spread of SARS-CoV-2 will rapidly overwhelm healthcare systems. A depletion in healthcare resources will lead not only to elevated COVID-19 mortality but also to increased all-cause mortality."[1220] And isn't that exactly what we have seen in the United States?

The Variability of Natural Immunity vs. the Stability of Vaccine Immunity

Whether vaccinated or infected, it takes time for the body to respond with the production of antibodies specific to the virus. In the vaccinated this occurs *before* infection and *without* COVID-19 illness. In the unvaccinated this occurs a*fter* infection and *with* COVID-19 illness. The time it takes for these antibodies to appear is variable, but typically within 2–3 weeks.

A significant complicating factor is that so-called natural immunity tends to be much more variable than that found with vaccination. In other words, as mentioned earlier, it is much more difficult to predict an individual immune response compared to the far greater homogeneity produced by vaccination.[1221] Put in slightly different terms, those who are vaccinated tend to look much more alike in their immune system profile (a relatively 'homogenous' response) than those who are infected (a 'heterogeneous' response). That means *one individual's immune response to a natural infection may confer better subsequent protection com-*

[1220] Randolph and Barreiro, "Herd Immunity," 729.
[1221] Baraniuk, "How Long Does Covid-19 Immunity Last?" 2.

pared to what he or she might have achieved by vaccination, but another individual's response might be far poorer.

There may be some advantages to the protection gained by infection rather than vaccination, but it is best understood in terms of *large groups* of people rather than individuals. A study reported in preprint examined natural infection versus vaccination by the mRNA vaccines (Pfizer's and Moderna's). It found that vaccination produces greater amounts of circulating antibodies than natural infection. On the other hand, actual infection may result in a greater advantage down the road when encountering virus variants. The study's authors suggest those vaccinated could profit, then, from subsequent booster shots.[1222] That is the same thought pointed out by the authors of the Israeli study.

In fact, the idea that those who have been infected can profit from subsequent vaccination and should be vaccinated has been shown in research done in this country. A study conducted in Kentucky found that unvaccinated survivors of COVID-19 are twice as likely to contract it again as people fully vaccinated after initially contracting the virus.[1223]

But given the higher variability among individuals who acquire immunity through infection, the caveat lies in *how* they acquire stronger immunity. Studies show those with higher initial antibody responses are more likely to have antibodies detected at a later date than those who had a lower initial antibody response.[1224] But how do those persons acquire these higher levels? Simply put, they had to be sicker in the first place. A study reported in late 2021 found that in an ethnically diverse cohort of COVID-19 patients, symptom severity correlated positively with higher antibody (IgG) production. Moreover, the longer a person was ill—usually with severe symptoms—the higher their subsequent antibody levels.[1225] *The sicker a person is the more likely they are to have a higher antibody count later.*

What are we left with? Even if it can be argued that natural immunity *might* provide superior protection at the population-level, individual outcomes still vary—and variability correlates with degree of illness. Achieving natural immunity comes only with infection, and may require several rounds of infection to become strong. It is hard to imagine even the most rabid anti-vaxxer being enthused at the prospect of severe and prolonged illness to acquire a high level of 'natural immunity.'

On the other hand, vaccination confers a more reliable immunity and appears to lessen risk of reinfection. Both alternatives may involve unpleasant and undesirable effects, but those associated with vaccination are typically fewer, rarer, and less serious than what occurs with natural immunity. Ultimately it seems those who make a choice against vaccines do so motivated by factors other than scientific facts.

[1222] Cho, et al., "Antibody Evolution." (This had not yet been peer-reviewed.)
[1223] CDC, "New CDC Study."
[1224] Self et al., "Decline in SARS-CoV-2 Antibodies," 1762.
[1225] Horton et al., "Determinants and Dynamics of SARS-CoV-2 Infection."

Perhaps it will prove to be the case that an anti-vax desire to have effective treatments will coincide with their development so that even if people elect not to get vaccinated there will be effective treatments widely available and at an early stage. An antiviral medication named *molnupiravir* is being developed and tested, as are others (e.g., PF-07321332, and AT-527), with some hope that one or more might be available by late Fall, 2021.[1226]

'Hybrid' Immunity

Breakthrough infections and reinfections—both more common with the advent of the Omicron variant—have led to a phenomenon some term *hybrid immunity*. This kind of immunity is characterized by two qualities:

- a measure of immunity provided by vaccination; and,
- a measure of immunity provided by infection.

In some instances infection precedes vaccination, as when a person who has experienced COVID-19 sensibly decides to seek the protection of vaccination. In other instances the vaccinated person experiences a breakthrough infection. Either way the result is hybrid immunity.

SARS-CoV-2 has proven to be adept at what immunologists term 'escape'—the ability of a virus to avoid immune system efforts at containment. Immunobiologist Emanuele Andreano and Rino Rappuoli, head of vaccine research and development at GlaxoSmithKline (GSK), use the metaphor of 'jail' in an instructive manner. The virus, quickly mutating, early proved its ability to escape the jail framed by natural immunity. Vaccines aim to securely cut off escape routes, thus building a better jail.[1227] Hybrid immunity, in this perspective, may represent the most secure jail.

Another analogy that has been offered comes from gardening. When different plant lines are bred together the resulting hybrid can be a stronger plant. It has been observed that something like this occurs when one line (natural immunity) is joined with another (vaccine immunity) against SARS-CoV-2. The result is *25–100 times higher antibody responses*. What is especially interesting is that this hybrid immunity cannot be attributed just to repeated exposure. (If that were true, one could argue that repeated infection or additional booster shots would work just as well, which does not seem to be the case.) The magnitude of immune response in hybrid immunization is much larger than simple repetition of some kind of exposure would warrant. Moreover, the degree of response also seems to be independent of how severe the natural infection was. (Remember, more severe infections tend to produce greater and longer immunity.) This synergistic effect has been likened to what is seen in the Shingrix vaccine against shingles, which uses vaccine to build upon previous infection that resulted in chickenpox.[1228]

[1226] Aleccia, "A Pill to Treat COVID-19."
[1227] Andreano and Rappuoli, "SARS-CoV-2 Escaped Natural Immunity," 760.
[1228] Croty, "Hybrid Immunity."

Studies of hybrid immunity consistently show it to be longer lasting than either immunity acquired through natural infection alone or through vaccination alone. For example, a study reported at the end of March, 2022, provided a comprehensive examination of the matter through a retrospective, total population cohort study in Sweden. This research involved three *cohorts* (groups sharing at least one important characteristic) to investigate natural immunity's protection against that provided by hybrid immunity. Thus one cohort's salient shared characteristic was immunity from infection with no history of vaccination while the other two cohorts also had a history of infection but with either one or two doses of a vaccine. The study looked at data for SARS-CoV-2 infection covering the period March 20, 2020–October 4, 2021. The following Table summarizes the cohorts and some of the study's findings.[1229]

	Cohort 1	**Cohort 2**	**Cohort 3**
Cohort Size	2,039,106	962,318	567,810
Type of Immunity	'Natural' (infection only)	'Hybrid' (infection + 1 vaccine dose)	'Hybrid' (infection + 2 vaccine doses)
Reinfection (at follow-up)	.08% (at 52 days) .04% (at 66 days) [1.67% (at 164 days)]	.27% (at 52 days)	.077% (at 66 days)
Lowered Risk of Reinfection *(Risk lowers with vaccination & booster)*	95% (after 3 months) *(against those with no immunity)*	58% *lower than natural immunity* (up to first 2 mos.) 45% *lower than natural immunity* (after 2 months)	66% *lower than natural immunity* (up to first 9 mos.)
Lowered Risk of Hospitalization	87% (up to 20 months) *(against those with no immunity)*		44% *(against those with natural immunity)*

The authors' focus was on natural immunity. They noted that natural immunity conferred modest but increasing protection against reinfection in the first three months, then substantially strong protection for up to 20 months. They also noted that natural immunity was associated with an *increased* risk of hospitalization in the first three months of follow-up compared to those with neither infection nor vaccination; if surviving that risk for three months, then

[1229] Nordström, Ballin, and Nordström, "Risk of SARS-CoV-2 Reinfection."

the odds in favor of escaping hospitalization increased dramatically and persisted up to 19 months.[1230]

With respect to the question at hand for us, the authors explicitly state, "In head-to-head comparisons, immunity acquired from a previous infection plus either one or two doses of a COVID-19 vaccine was associated with a greater reduced risk of SARS-CoV-2 reinfection and COVID-19 hospitalization for up to 9 months. . . ."[1231]

Another study implicitly contrasted hybrid immunity with simple reliance on natural immunity. It examined 63 individuals (ages 26–73, median age 47; 57% male) who had survived a verified case of COVID-19 and who were then assessed at 1.3, 6.2, and 12 months after infection. Of the 63 persons, 26 (41%) subsequently were vaccinated with an mRNA vaccine. Among those who relied only on natural immunity, the difference seen in their plasma for neutralizing activity against the virus at 12 months was about the same as at 6.2 months. But for the vaccinated, plasma neutralizing activity was *almost 50-fold higher* than that of the unvaccinated. Among those who relied only on natural immunity, neutralizing activity against variants was lower; for the vaccinated such activity increased. With respect to memory B-cells that produce antibodies neutralizing the receptor-binding domain (RBD) of SARS-CoV-2, in the unvaccinated levels at 12 months after infection were 1.35-fold lower than they had been at 6.2 months. By contrast, for those vaccinated the levels showed an average 8.6-fold increase.[1232]

Clearly, hybrid immunity provides superior protection. It has been compared to being like an additional booster shot—a fourth dose—against the virus. One virologist, Shane Crotty at the La Jolla Institute of Immunology, commenting about its effectiveness against SARS-CoV-2, remarked, "It's the best immunity you can get. But I wouldn't think of hybrid immunity as being a force field that can completely stop it no matter what."[1233]

That caution is significant. Remember, natural immunity from infection is highly variable. Unfortunately, the more severe the infection the more robust the subsequent immunity—provided one survives the experience. And even if one does, the significant risk of long COVID looms. In short, no one in their right mind should be eagerly seeking infection in hope of acquiring some kind of super immunity.

At the same time, though vaccination provides excellent and stable protection, it also is limited in nature. But forced to choose between natural infection and vaccination, the latter is by far the safer alternative. Then, if a breakthrough infection does occur, the odds are strongly in the person's favor of less severe outcomes—and then the added benefit of hybrid immunity.

[1230] Nordström, Ballin, and Nordström, "Risk of SARS-CoV-2 Reinfection," 6.
[1231] Nordström, Ballin, and Nordström, "Risk of SARS-CoV-2 Reinfection," 7.
[1232] Wang et al., "Naturally Enhanced Neutralizing Breadth against SARS-CoV-2," 427.
[1233] Quoted in Blum-Friday, "How Does Getting a Breakthrough Covid Case Affect My Immunity?"

SARS-CoV-2 is a formidable foe. Researchers Marta Ferreira-Gomes and colleagues begin an article study by remarking, "One of the properties of SARS-CoV-2 which makes it spread so successfully, is the heterogeneity of human immune responses it triggers—from asymptomatic to fatal. However, the molecular reasons for this heterogeneity of COVID-19 are not clear."[1234] In somewhat simpler terms, the enemy somehow fetches from us highly variable immune responses—and we really don't know why.

But there are some things that have become quite clear. Researchers Nicholas Murgolo and colleagues note, "Immune dysfunction has been extensively characterized in COVID-19 patients, such as dysregulation of T cells, B-cells, and innate immune cells."[1235] We know that whatever it is doing, SARS-CoV-2 interacts with us in ways that very frequently result in very messed up immune system responses. Put bluntly, only a fool relies exclusively on natural immune system protection.

Anti-vaxxers regularly sing the praises of the human body's immune system. The praise is justified; our immune responses are marvelous and often life-saving. But the immune system is not infallible and viruses are proficient at evading it and even exploiting it. Even at its best, the immune system takes time to mount an effective resistance to SARS-CoV-2. It can take days—even weeks—for measurable amounts of antibodies to be developed to fight COVID-19. The virus cares not at all about the so-called benefits of 'natural immunity'—all the while the body is gearing up the virus is active, spreading itself and its harmful effects. The goal of vaccines is to assist the human immune system by preparing it while the stakes are low—that is, before an actual infection, with all its potential harm, has occurred.

[1234] Ferreira-Gomes et al., "SARS-CoV-2 in Severe COVID-19," 2.
[1235] Murgolo et al., "SARS-CoV-2 Tropism," 11.

Chapter 7
Vaccines

Let's start our consideration of vaccines by returning to a concern of the last chapter—the question of whether vaccines or natural infection provide better immunity. This time let us frame the matter more broadly than just with respect to SARS-CoV-2. The following Table provides a snapshot with regard to the length of immunity for a dozen well-known pathogenic agents as measured by persistence of detectable antigen-specific antibodies.[1236]

Infectious Agent	Kind B= bacteria V= virus	*Persistence of Antibodies (half-life mean)*	Infectious Agent	Kind B= bacteria V= virus	*Persistence of Antibodies (half-life mean)*
Chickenpox[1]	V	*50 years*	Mumps	V	*life-long*
Diptheria	B	*19 years*	Polio	V	*40 years*
Epstein-Barr (EBV)	V	*life-long*	Rubella[2]	V	*life-long*
Hepatitis A	V	*25 yrs-life*	Shingles[3]	V	*50 years*
Measles	V	*life-long*	Tetanus	B	*11 years*
Meninco-goccal	B	*3–5 yrs.*	Yellow Fever	V	*75 years*

[1] *Varicella-Zoster* virus [2] Aka. 3 day measles, or German measles. [3] *Varicella-Zoster* virus

The above Table reflects averages (the mean) for how long it takes for the concentration of antibodies specific to the infectious agent to decrease by half (half-life). Not all the infectious agents listed above are viruses, but generally speaking antiviral antibody responses prove very stable. The varicella-zoster virus that causes chickenpox and shingles is a DNA virus, as is EBV. On the other hand, the viruses that cause hepatitis, measles, mumps, polio, rubella, and yellow fever are all RNA viruses. As we learned earlier, DNA viruses are larger and less prone to mutations, making them relatively easier to immunize against.

But the kind of virus is only one factor that affects the length of protection achieved by natural infection or vaccination. Most notably, reexposure to the antigen is important in keeping the body's immune defenses active. However,

[1236] Table based on various sources, including Ammana, Carlson, and Slifka, "Duration of Humoral Immunity," 1906 (Table 2); Cohen and Burbelo, "Reinfection with SARS-CoV-2," e4224 (Table 1); Sifka and Ahmed, "Long-term Humoral Immunity against Viruses," 395 (Table 1).

the authors of one study concluded that individual variation in maintaining antibodies is only partly determined by reexposure to antigen; other factors involved cannot be easily determined.[1237] Because of this complexity, as noted elsewhere in this book, the degree of protection—'immunity'—is variable. The figures in the Table should not be read as some kind of guarantee for every individual.

Let's focus for a moment on the RNA viruses. The preceding Table does not differentiate between whether the degree of immunity was achieved through natural infection or via vaccination. But should we assume immunity by either route is the same? As our previous discussion of the matter indicates, we should *not* make such an assumption. The history of diseases and vaccinations demonstrates there can be a difference in protection between that provided by infection and that by vaccination. So let's adjust our Table and look at that matter.

RNA Virus	Natural Immunity	*Vaccine Immunity*	RNA Virus	Natural Immunity	*Vaccine Immunity*
Hepatitis A	life-long	*20+ years*	Polio	40+ years	*unknown*
Measles*	life-long	*life-long*	Rubella*	life-long	*life-long*
Mumps*	life-long	*16–51 yrs. (M=27 yrs.)*	Yellow Fever	life-long	*life-long*

* Measles, Mumps, Rubella combined in MMR vaccine. [2] Aka. 3 day measles, or German measles.

Let's start with *hepatitis A*, which is perhaps the most problematic of this group. Because, as we have seen, immunity results in different degrees in different people for different reasons, neither for hepatitis A nor any other virus listed above should we state unequivocally that infection or reinfection is impossible. With hepatitis A the persistence of antibody can be different than the length of actual immunity. In practice, it is thought that infection results—*typically*—in life-long protection. For the vaccine, the actual duration of immunity is unknown, but the evidence suggests *at least* 20–25 years.[1238] So the apparent difference shown in the Table may not be as pronounced as it seems.

As the Table indicates, immunity to *measles* is considered life-long. But that for SARS-CoV-2 is proving to fall far short of such duration. Although both are RNA viruses, the measles virus (MeV) is rather exceptionally different. It has been noted that antigenic drift and genetic variation are significantly constrained in MeV and it exhibits high genetic stability.[1239] This relative lack of mutating means that defenses mounted against MeV continue to be effective. *Rubella* and *congenital rubella syndrome* (CRS)—commonly grouped together with measles in

[1237] Ammana, Carlson, and Slifka, "Duration of Humoral Immunity," 1911.
[1238] For a start on this topic, see CDC, "Hepatitis A Questions and Answers."
[1239] Beaty and Lee, "Constraints on the Genetic and Antigenic Variability of Measles Virus," 11.

discussion and also in one vaccine—were both declared eliminated in the United States in 2004 and the region of the Americas in 2015.[1240]

Mumps is a slightly different story. Also included in the measles-mumps-rubella (MMR) vaccine, it is generally considered to provide life-long protection in many people, though it wanes over time. The risk comes for those exposed to someone with an infection. At-risk people have immunity restored with a booster shot.[1241]

Polio (*poliomyelitis*) presents a significant complication: it exists in three types (P1, P2, and P3). Contracting infection of one type can provide life-long protection from that type, but does not offer immunity against the other types. Early in the 21st century, only seven countries in the world showed a detectable level of polio. The near-eradication of polio has been the result of vigorous vaccination.[1242] The tremendous success, though, is owed in part to the fact that—like measles—the polio virus is highly stable genetically and does not mutate much. The protection of the vaccine is listed as unknown, because of a lack of sufficient evidence to say otherwise, but is thought to confer life-long immunity.

Yellow Fever is a mosquito-borne disease. Both natural immunity and vaccine immunity (provided by a single dose) are life-long,[1243] but the latter is without the serious risks associated with being infected and experiencing the disease.

Why are vaccines so successful against these RNA viruses? There is no one-size-fits-all answer. For example, as mentioned above, both the measles and polio viruses are very genetically stable; less mutating means vaccines remain highly viable. With hepatitis A the virus replicates slowly, which provides the body opportunity to engage its defenses more effectively. The vaccine, which is antibody-based, thus advantages itself of this viral feature. But it also exhibits only one serotype, meaning its surface antigens present a regular, predictable presentation that also simplifies the work of a vaccine. [1244] Rubella also presents just one serotype, and its two genotypes are very, very similar (1–3% different). It has a generally long incubation period and rubella antibodies show excellent persistence—all factors that help the body and vaccines succeed.[1245] In short, a variety of fortunate factors help vaccines succeed. As the Table shows, vaccines against the viruses listed are quite comparable to the immunity provided by natural infection—and without the attendant risks.

But not all RNA viruses are as amenable to vaccination. Fortunately, this is not as serious issue as one might fear both because the virus does not often produce serious consequences, and/or the vaccine's degree and duration of pro-

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[1240] PAHO, "Americas Region Is Declared the World's First to Eliminate Rubella."
[1241] For a reasonably short overview of the issues, see CDC, "Mumps Vaccination."
[1242] WHO, "Poliomyelitis."
[1243] PAHO, "Yellow Fever" ; WHO, "Yellow Fever."
[1244] For an overview of hepatitis A, see Martin and Lemon, "Hepatitis A Virus"; for its genetic variability, see Costa-Mattioli et al., "Genetic Variability of Hepatitis A."
[1245] For an overview of rubella, see Lee and Bowden, "Rubella Virus Replication and Links to Teratogenicity." Also see Plotkin, "Rubella Eradication: Not Yet Accomplished."

tection is sufficient as weighed against the health risk from infection. The following Table offers a quick glance at some of the RNA viruses that have proven successful at immune escape, thus affecting vaccines negatively. Remember, RNA viruses often experience rather rapid mutations in just the ways designed to frustrate host cell defenses.

RNA Virus	***Presumed Immunity***	**RNA Virus**	***Presumed Immunity***
Influenza*	*30 months*	Rotavirus	*12 months*
Coronaviruses	*12 months*	SARS-CoV-2	*(being determined)*

* For variants covered by the annual vaccine.

Now the first thing that must be said is that a virus that escapes what a vaccine does in defense is also going to escape what a human body does in defense. Vaccines do not replace the immune response, but prime it. Thus failure to any degree in a vaccine likely reflects a similar problem at the level of the immune system.

Our chapter on the immune system provides brief coverage of the various challenges the immune system faces with viruses that continually change. But that coverage is somewhat misleading in the sense that, as declared at its start, it accentuates the positive. The truth of the whole matter is far messier—as the subsequent chapter specifically on the war against SARS-CoV-2 points out. As good as it is, the immune system is not perfect and pathogens like viruses have learned to exploit its vulnerabilities no less than computer viruses successfully hack and take over computer systems. The immune system is in a constant struggle to keep up with, let alone stay ahead of, things like viruses.

The immune system's basic philosophy is that it's better to suffer than die, and better by far to sacrifice some cells so the whole body will survive. It has a blunt object approach to successfully invading pathogens—bludgeon and destroy. If it can eat a pathogen, well and good; but often it resorts to burning it out—inflammation—a sort of scorched earth policy that wins wars but leaves the countryside in poor shape. In fact, sometimes the effort at a 'natural cure' proves fatal as inflammation runs out of control.

Still, we can, as some suggest, let Nature take its course and rely on the immune system as it is, just as we can we opt to walk rather than trust man-made vehicles, or cook all our food over an open fire rather than microwave it or use an oven. To each their own. But most of us find many human achievements practical and praiseworthy, from planes, trains, and automobiles, to indoor plumbing, electricity, and stoves. Vaccines are a human endeavor to artificially aid natural processes, just as many other technological advances are. But just as any tool should be properly understood as to its intent, function, and correct use, so should vaccines.

Many find it remarkable that vaccines are controversial given their long history of success. But because of that success too many have forgotten what things were once like in the world.

A Context for Understanding Vaccine History

Historian of medicine Allan Brandt observes that as the 20th century dawned there was a shared sense of vulnerability created by the previous two centuries having experienced widespread occurrences of serious epidemics. He writes, "Epidemics, with their sudden onset and often disastrous impact, created a perception of a world not clearly under human control. Efforts to arrest these contagions through sanitation, quarantine, or medical intervention often failed to have any influence on the course of disease."[1246]

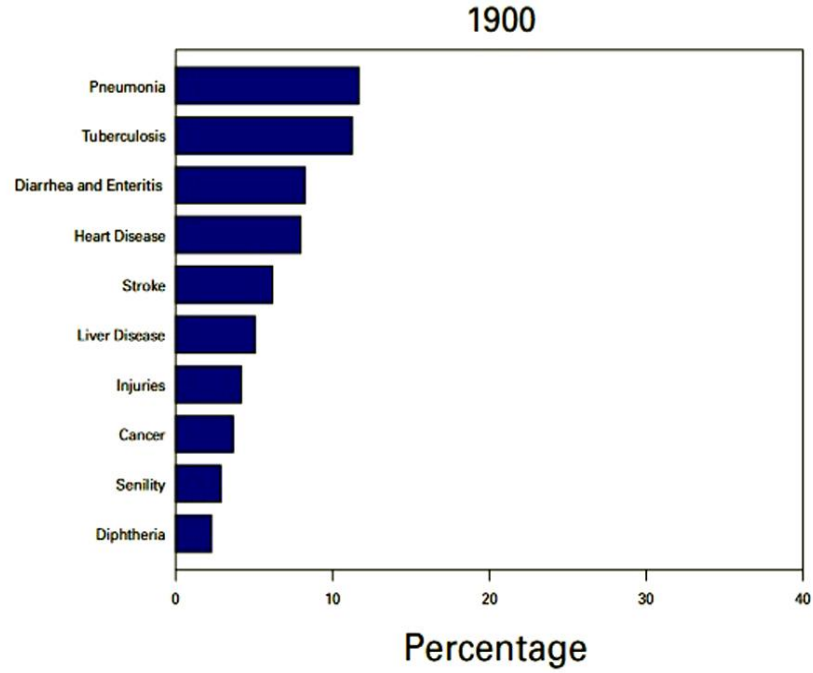
By the time the 19th century closed the toll taken by infectious diseases was both apparent and appalling. Estimates of the percentage of deaths in the world's reining power—Great Britain—were that 33–40% of all deaths in that century were attributable to such diseases.[1247] For example, in 1850, 44.7% of deaths in England and Wales were attributed to infectious diseases, and a half-century later that figure was still 35.8%.[1248] In the United States, in 1890, the leading cause of death was 'consumption' (tuberculosis) accounting for 121.49/1,000 known causes of death (12.15%). Leading causes of death by infectious disease included diarrheal diseases (e.g., cholera and dysentery; 8,533/100,000), diphtheria (3,177/100,000), typhoid fever (3,091/100,000), malaria (2,124/100,000), and measles (1,057/100,000).[1249]

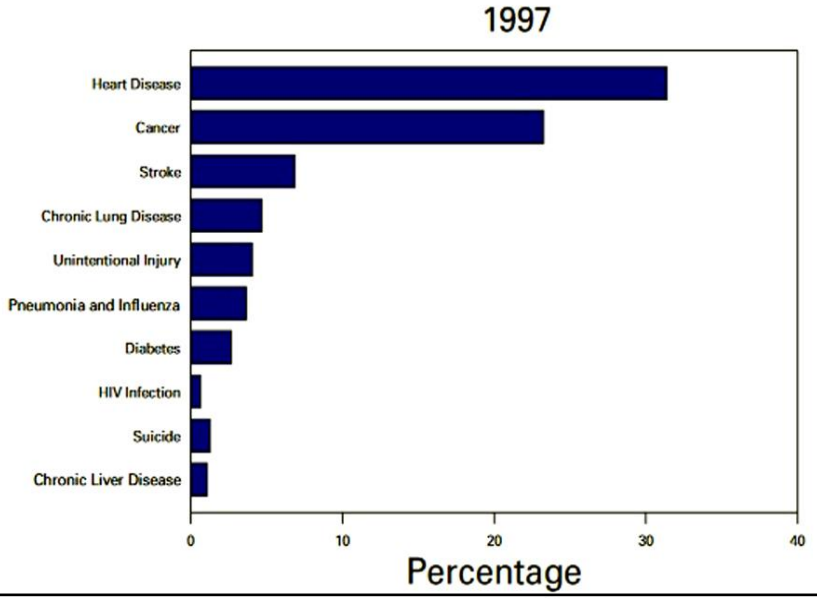
In 1900, life expectancy in the United States for both men and women was less than 50 years. A major factor for this reality was the widespread existence of diseases such as smallpox, diphtheria, tuberculosis, typhoid fever, pertussis ('whooping cough'), tetanus, measles, and pneumonia, among other scourges. Although slow advances in hygiene, cleaner water, antibiotics, and medical advancements all eventually contributed to diminishing these threats, science largely credits vaccines for the greatest role in limiting or eliminating the above listed causes of death—for each of which a highly effective vaccine exists.[1250]

In 1900, the three leading causes of death in the United States were pneumonia, tuberculosis (TB), and diarrhea and enteritis, which (together with diphtheria) caused one third of all deaths.[1251] In 2020, the three leading causes of death in the United States were heart disease, cancer, and COVID-19.[1252] But this contrast might be better seen in the illustration on the next page.

[1246] Brandt, "Behavior, Disease, and Health in the Twentieth-Century United States," 54.
[1247] Condrau and Worboys, "Second Opinions," 166.
[1248] Shaw-Taylor, "An Introduction to the History of Infectious Diseases," E5 (Table 2).
[1249] Billings, *Report on Vital Statistics in the United States at the Eleventh Census: 1890*, 223–24.
[1250] Barocchi, Black, and Rappuoli, "Multicriteria Decision Analysis and Core Values," 1.
[1251] "Control of Infectious Diseases," 621.
[1252] Ahmed and Anderson, "The Leading Causes of Death in the US for 2020."

FIGURE 2. The 10 leading causes of death as a percentage of all deaths — United States, 1900 and 1997





Material courtesy of "Control of Infectious Diseases." *Morbidity and Mortality Weekly Report* 48/29 (July 30, 1999), p. 623. This work is in the public domain.

Let simple, stark before-and-after numbers tell their story. Figures presented below represent rates in times slightly before a vaccine was created while the numbers after reflect the impact of vaccination over the period since.

PATHOGEN	Before Vaccination	After Vaccination
Diphtheria[1253] (Antitoxin, 1890s; vaccine developed 1920s; widely used in the early 1930s. Added to tetanus toxoid and pertussis vaccine, wide use 1940s.)	**79.4/100,000** (20 nations, 1889–1896, *before antitoxin*)	**16.3/100,000** (20 nations, 1907–1914 *after antitoxin*; 63% lower)
	121.9/100,000 (12 major U.S. cities, 1885–1894)	**28/100,000** (12 major U.S. cities, 1885–1894; 93.9% reduction)
	100–200,000 cases annually, with 13,000–15,000 deaths (U.S., 1920s before vaccine use)	14 cases, 1 death (U.S., 1996–2018)
Smallpox[1254] (1st vaccination in 1796.)	Estimated 400,000 dead annually at end of 18th century.	"We, the members of the Global Commission for the Certification of Smallpox Eradication, certify that smallpox has been eradicated from the world." (1980)
Tuberculosis[1255] (Vaccine developed 1921.)	**800-1,000/100,000** (in Europe, North America and U.S., 19th cent.);	**40.09/100,000** (worldwide, 1990); **14.64/100,000** (world-(wide, 2019)
	194/100,000 (U.S., c. 1900)	**3/100,000** (U.S., 2021, approx.), with 526 deaths.
Typhoid Fever[1256] (1st vaccine developed 1896; Ty21a vaccine developed in early 1970s)	Common, but highly variable mortality rates in 19th century	**0.13-1.2/100,000** (developed nations, early 21st cent.);
	31.3/100,000 (U.S., 1900)	**1.1/100,000** (U.S., 1933)
		35,000 cases annually (U.S., 1920s); 300-400 cases annually (U.S., 1980s)

[1253] Crum, "A Statistical Study of Diphtheria," 446 (Table 1) (before and after antitoxin); Acosta et al., "Diphtheria," 97, 102 (U.S. before and after). Also see Truelove et al., "Clinical and Epidemiological Aspects of Diphtheria."

[1254] Behbehani, "The Smallpox Story," 458 (before) and 499 (after).

[1255] Daniel, "The History of Tuberculosis," 1864 (Europe, NA, and U.S., before); Murray, "A Century of Tuberculosis," 1181 (U.S., before); *Our World in Data* "Deaths-Tuberculosis" (world, after) (https://ourworldindata.org/grapher/tuberculosis-death-rates?tab=table), and see Fukunaga et al., "Epidemiology of Tuberculosis," 427 (Table); CDC, "TB in the United States, 2021" (U.S., after).

[1256] On the vaccine, see Marathe et al., "Typhoid Fever & Vaccine Development"; Collins, "History and Frequency of Typhoid Fever," 897; Adler and Mara, *Typhoid Fever: A History*, 192, 194 (U.S., after).

Tuberculosis (TB) remains the biggest challenge; where widespread vaccination has prevailed incidence and mortality rates have declined dramatically. But in regions of the world where this is not the case—and complicated by HIV—the results have been concerning (see Table below). TB ranks second only to COVID-19 as an infectious cause of death.

TABLE. Estimated number of incident tuberculosis (TB) cases, TB incidence rate, number of TB-associated deaths among all persons and among HIV-positive persons, and number of TB patients with rifampicin-resistant TB (RR-TB), by World Health Organization region — worldwide, 2019

WHO region	No. of TB cases, x1,000	Incidence*	No. of deaths, x1,000 (CFR, %)	No. of TB cases among HIV-positive persons, x1,000	No. of TB deaths among HIV-positive persons, x1,000	No. of RR-TB cases,† x1,000	Incidence of RR-TB*,†	% of RR-TB cases†
Global (all regions)	**9,960**	**130**	**1,418 (14.2)**	**815.0**	**208.0**	**465**	**6.1**	**4.7**
African	2,470	226	547 (22.1)	595.0	169.0	77	7.0	3.1
Americas	290	29	22.9 (7.9)	29.0	5.9	11	1.0	3.8
Eastern Mediterranean	819	114	78.7 (9.6)	7.9	2.7	36	5.0	4.4
Europe	246	26	24.2 (9.8)	30.0	4.2	70	7.5	28.5
South-East Asia	4,340	217	652 (15.0)	117.0	20.0	171	8.6	3.9
Western Pacific	1,800	93	90.3 (5.0)	36.0	6.3	101	5.2	5.6

Source: Adapted from World Health Organization. Global tuberculosis report 2020. Geneva, Switzerland: World Health Organization; 2020. https://www.who.int/teams/global-tuberculosis-programme/tb-reports
Abbreviation: CFR = case-fatality rate.
* Number of cases per 100,000 population.
† Includes multidrug-resistant TB.

Material courtesy of Fukunaga, Rena, et al. "Epidemiology of Tuberculosis and Progress toward Meeting Global Targets—Worldwide, 2019." *Morbidity and Mortality Weekly Report* 70/12 (Mar. 26, 2021) 427–30. doi: 10.15585/mmwr.mm7012a4, p. 427. This material is in the public domain.

An Abbreviated History

The history of vaccines may have older antecedents but its beginning is generally placed at the development of the vaccine for smallpox. Most people at some point have likely heard of the work of Edward Jenner (1749–1823), who administered his first smallpox vaccination in 1796.[1257] Jenner's work was set against a background of medical practice to treat smallpox by *variolation*—intranasal inhalation of pox material, which produced infection, but generally of a mild form and resulted in protective immunity. This had been normative practice for a half-century when Jenner began his medical career, and he also practiced it. But as a country doctor tending to farmers he encountered a number who had contracted a disease from milking cows—'cowpox'—a skin disease similar to smallpox, but milder. Those who had experienced this disease were subsequently immune to smallpox. Based on his observations and careful records, after the passage of a number of years, he performed his first vaccine inoculation on a boy of about 8, who subsequently proved immune to smallpox.[1258] Some 180 years later, the disease was declared eradicated.

Diseases like smallpox, left to spread through populations to achieve 'natural immunity,' can persist for centuries as widespread and deadly entities. Here is a simple historical observation: *if viruses aren't contested they don't go away*. There are different ways to contest viral spread of disease—healthy habits and isolation from large groups, for example. But only one method has ever succeeded in

[1257] Baxby, "Edward Jenner's Role," 13.
[1258] Smith, "Edward Jenner and the Small Pox Vaccine."

eliminating a viral threat—and that is vaccination. The example of smallpox is significant, though also cautionary. Vaccination progressively reduced smallpox's power by achieving a degree of herd immunity that natural means never achieved. But the nature of smallpox (a DNA virus rather than an RNA virus) made it less versatile and thus more amenable to elimination. RNA viruses, like SARS-CoV-2 mutate rapidly and are thus harder to eliminate.

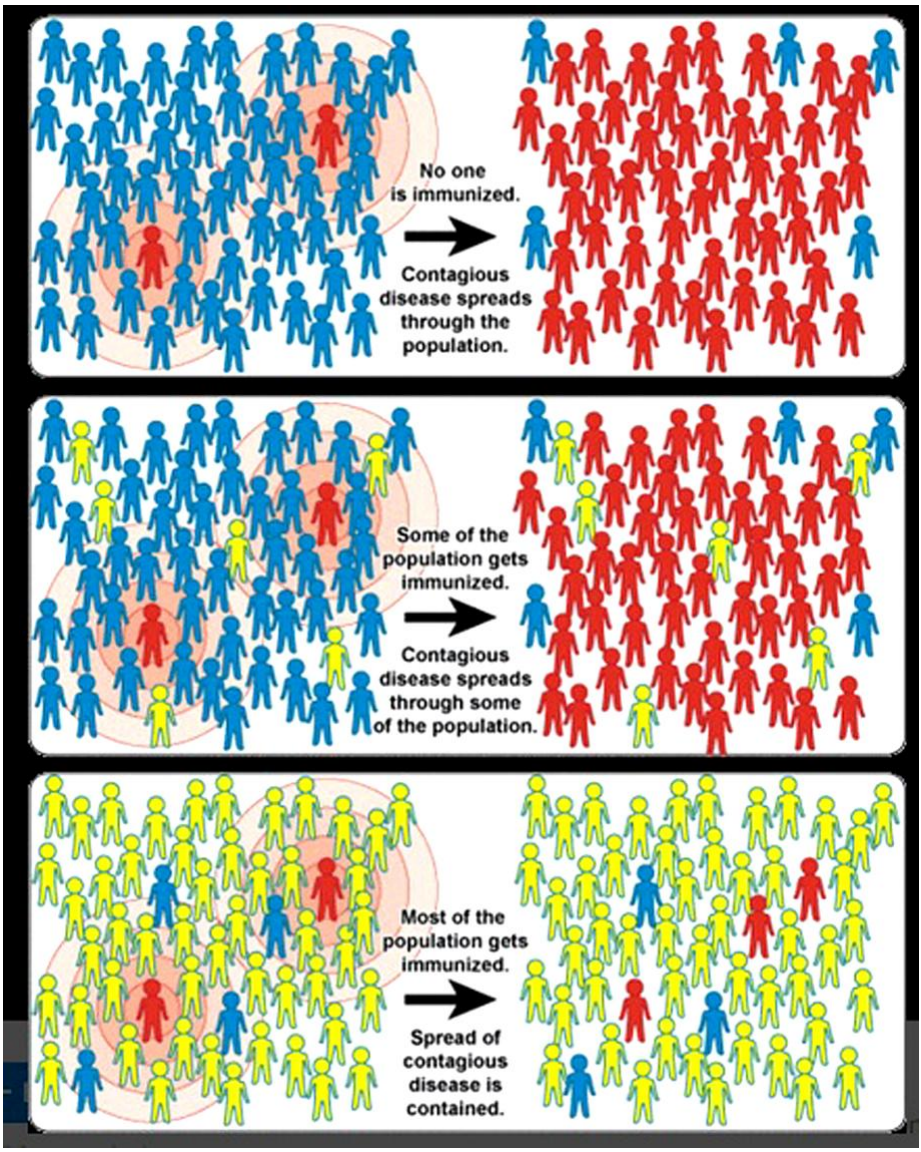
It is unreasonable to expect vaccination programs to achieve against every disease agent the degree of success found with smallpox. But that does not mean vaccines have not been spectacularly successful. Other major pathogens—polio and rabies—have been largely brought under control through vaccines.[1259] The World Health Organization (WHO) estimates, for another example, that just *in the first 15 years of the 21st century, some 17.1 million lives had been saved by vaccination from the disease of measles*.[1260] But measles vaccine is just one success story among many. Medical reporter Tish Davidson, in her history of vaccines, points to the success against a disease little noted today. "In the 1920s," she writes, "before an effective diphtheria vaccine became available, at least 200,000 people in the United States contracted the disease each year. Between 10,000 to 20,000 of them died. The death rate in children under five was 20 percent. Now sometimes years go by without a single case in the United States."[1261] Large books document the success of vaccines, and even a cursory comparison of countries today where vaccination and good sanitation are widespread, as opposed to those where these remain uncommon, shows tremendous differences in health outcomes. For example, in 2017 the incidence of typhoid/paratyphoid fever in central and eastern Europe and central Asia ranged from 0.1–0.7/100,000, but in sub-Saharan Africa (eastern and western) it ranged from 151.9–161.1/100,000 (respectively). It was 549.2/100,000 in South Asia.[1262]

That vaccines are questioned by as many people as seems the case is principally due to fear-mongering perpetrated by profit-minded anti-vaxxers who capitalize on a science-ignorant public who hold particular cultural values. Cynical anti-vaxxers and populist politicians take advantage of certain beliefs by attaching their anti-vaccination claims to them (whether warranted or not).[1263]

In 2012, then editor of the *Virtual Mentor* (*American Medical Association Journal of Ethics*) Michael Janko remarked, "We question the value of vaccines because they have become a victim of their own success, so it is an imperative that we educate ourselves with objective, fact-based information to weigh the benefits and risks of vaccination."[1264] A decade later, still struggling to emerge from a pandemic that has taken over a million American lives and more than six million worldwide, those sensible words ring even more urgently.

[1259] Kayser and Ramzan, "Vaccines and Vaccination: History and Emerging Issues," 5225.
[1260] WHO, "Measles Vaccination Has Saved an Estimated 17.1 Million Lives Since 2000."
[1261] Davidson, *Vaccines*, xi.
[1262] GBD 2017 Typhoid and Paratyphoid Collaborators, "The Global Burden," 373 (Table 2).
[1263] For a full treatment of this matter, see Bolich, *Understanding COVID-19 Vaccination Arguments*.
[1264] Janko, "Vaccination: A Victim of Its Own Success," 4.

The illustration below, courtesy of the National Institutes of Health (NIH), tells the story of immunization by vaccines in a nutshell.



[Key: Red—infected person; Blue—unvaccinated, uninfected person; Yellow—vaccinated person.]

All sensible people praise the virtues of the human immune system, but the above illustration suggests that left alone, a pathogen like a virus—for example, SARS-CoV-2—prevails against the immune system and spreads far and wide. Vaccines help prevent this spread *and* protect individuals.

Let's be clear what vaccines are about—*prevention before* the fact of damage rather than treatment after it, and *limitation of damage if it does occur* rather than scrambling to corral the horse after let out of the barn. Anti-vaxxers spread fear about the process of vaccine disease prevention and promote focus instead on what they want consumers to buy—their treatments. Those in the business of both prevention and treatment, like the pharmaceutical company Pfizer, which provides a vaccine for COVID-19 and also has developed a pill to treat the disease, understand the desirability of vaccines as being the frontline defense against disease. In an interview given in December, 2021, Pfizer CEO Albert Bourla said, "Vaccines [are] the primary frontier that you should be using to stop the disease. The goal is not to get sick and then hopefully have a medicine that can save you. The goal is not to get sick."[1265] The logic really is that simple: focus on avoiding getting sick rather than waiting until it happens and then popping a pill.

A Philosophy for the Task of Vaccination

Nevertheless, though the basic philosophy of vaccines is simple enough, the process of bringing about a vaccine that works well and safely is quite complicated. To craft a philosophy for the task of vaccination—a task with many stakeholders all wanting their interests represented—is an ongoing process.

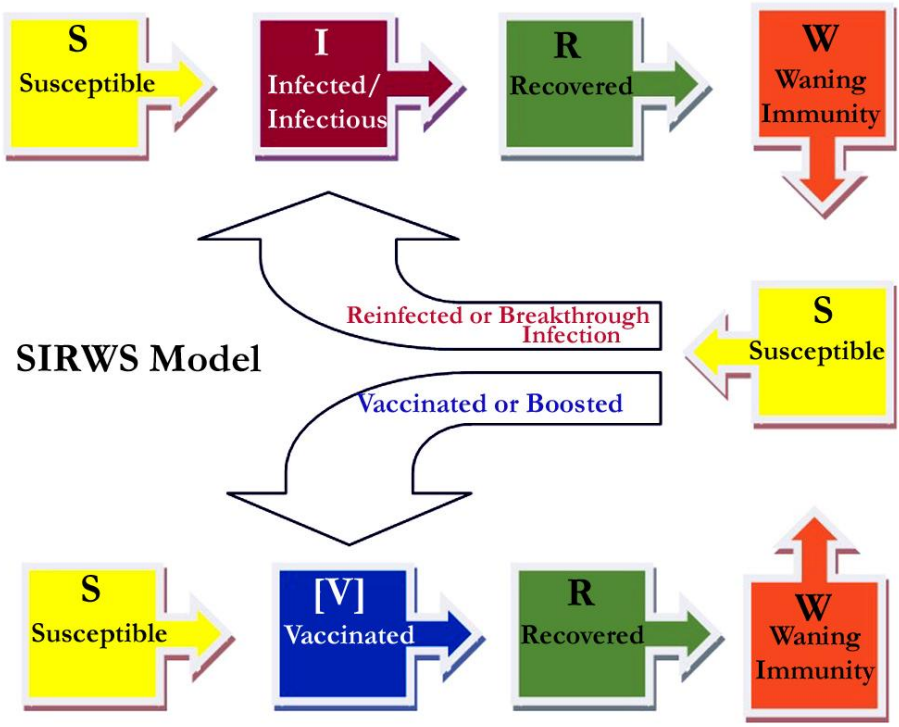
In the United States, organizational bodies like the U.S. National Academy of Medicine (NAM) have spent decades crafting and refining policy recommendations with respect to vaccines. In the early 21st century a working group in NAM proposed using a multicriteria decision-making process for vaccines, and then developed a software tool for it—*Strategic Multi-Attribute Ranking Tool* (SMART). This development inspired a 2015 conference in Siena, Italy, where major stakeholders from diverse arenas (e.g., science, public policy, health economics, and vaccine manufacturing) gathered to consider the feasibility of using many criteria rather than just cost-effectiveness (a traditional standard). They also considered 28 vaccine attributes, grouped into 8 categories, that NAM had proposed, in order to see if a set of core values could be formulated. The result was to recommend employing a multicriteria decision-making process reflecting three core value considerations:

[1] mortality and severity of the disease;
[2] vaccine safety considerations, and
[3] economic evaluation that captures the full benefits of vaccination.[1266]

Of course, each value consideration reflects exactly that—*values*, things people esteem highly and to which they are prone to attach moral judgments. Thus a workable philosophy is always one reflecting values in a practical context.

[1265] Albert Bourla, quoted in Takala, "Pfizer CEO Warns Patients."
[1266] Barocchi, Black, and Rappuoli, "Multicriteria Decision Analysis and Core Values."

Part of that practical context is comprehending patterns of infection and the differences existing between two paths: natural infection and intervention through vaccination. A common procedure to mathematically understand the epidemiological patterns of infectious disease is to utilize the *susceptible-infectious-recovered-susceptible* (SIRS) *model*. This basic model can be modified by including waning and boosting of immunity to become a *susceptible-infectious-recovered-waning/boosting-susceptible* (SIRWS) *model*. The latter, with its inclusion of immune boosting, produces a model that can exhibit periodic cycles when differences between natural- and vaccine-acquired immunity exist. This helps in better seeing how infection prevalence changes with vaccination coverage and duration of immunity. Researchers using this model have shown, as they state, "the main contributor to the force of infection (and thus transmission) depends on a complex interplay among the level of vaccination coverage, the duration of infection- and vaccine-acquired immunity, and the mechanism through which immune boosting provides protection (delaying or bypassing a primary infection)."[1267] Let's begin to picture this.

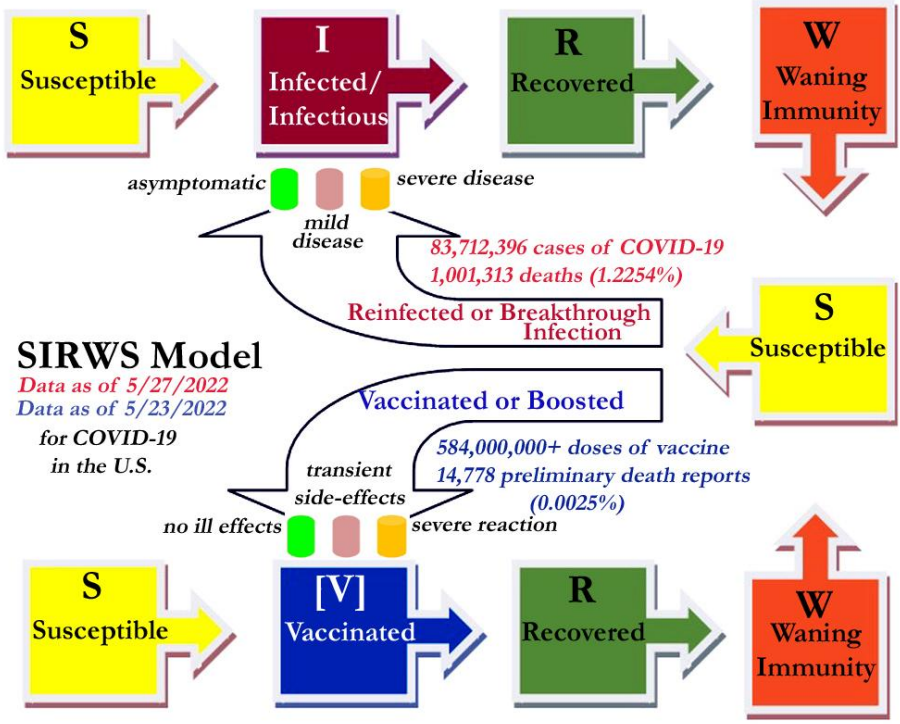


Everyone starts at the left—'susceptible'—though because of a number of factors that susceptibility is variable. Two paths then quickly emerge: one is vac-

[1267] Leung et al., "Infection-Acquired Versus Vaccine-Acquired Immunity," 119, 126 (quote). Their model assumes a vaccine providing the same protection as infection, except for duration.

cinated or infected. Of course, some lucky few are neither; they avoid infection even though unvaccinated. But such outliers can be set aside here since our interest is in seeing the difference between vaccination and infection (assuming, in each instance, survival). Both paths lead to recovery, with some degree of immunity, and both paths lead to waning immunity, followed by again some degree of susceptibility. At that point another divergence occurs. Whether previously conferred a degree of immunity by infection or vaccination, the person now susceptible might regain robust immunity through infection/reinfection, or vaccination/vaccine booster. Individuals can switch the path they are on.

Superficially, it may seem there is no appreciable difference between the paths of natural infection and vaccination; both lead to immunity, which wanes, and makes one susceptible again. But let's adjust the picture in real-world terms of risks associated with each path with respect to COVID-19. The adjusted illustration below categorizes risks but reports only deaths in the United States[1268] (see elsewhere in this book for information related to the categories).



[1268] Data on cases of infection and death from CDC Tracker (https://covid.cdc.gov/covid-data-tracker/#datatracker-home) as of May 27, 2022. Data on vaccines and deaths reported from CDC, "Selected Adverse Events Reported after COVID-19 Vaccination" (https://www.cdc.gov/coronavirus/2019-ncov/vaccines/safety/adverse-events.html), as of May 23, 2022.

It doesn't take any genius in mathematics to see that 0.0025% is very, very much smaller than 1.2254% (about 490 times smaller). The good news is that both are small numbers, but *comparatively* the risk of the most adverse outcome—death—is far greater from the path of natural infection compared to that of vaccination. Furthermore, as documented elsewhere in this volume, the possible adverse outcomes from vaccination are fewer, rarer, and generally of less consequence than those from natural infection. As to the degree of protection acquired, as we have seen it is highly variable for those who choose natural immunity, and more reliably the same across those who choose vaccination.

So let us now back out to look at the bigger picture once again. As a general rule, vaccines provide long-lasting protection, but natural infection provides even longer immunity. But if evaluated from a risk-reward perspective, the matter rather simply looks like this:

	Natural Infection	**Vaccination**
RISK of Adverse Outcome	much higher	much lower
REWARD of Robust Immunity	variable	stable and high
Duration of Immunity	longer	shorter

The potential reward of longer immunity is associated with the risk of a more likely and more severe adverse outcome. Is the reward worth the risk?

More people are risk-averse when the goal is loss-aversion. Psychologist Russell Poldrack, who has studied the neurobiology of the matter, explains, "[T]he brain regions that process value and reward may be silenced more when we evaluate a potential loss than they are activated when we assess a similar-sized gain."[1269] In other words, the potential reward needs to be substantially larger than the potential loss to adequately tempt us to take the risk. This helps explain why, despite much expressed vaccine hesitance in the United States, by late May, 2022, more than 80% of people ages 5 and up had received at least one dose of vaccine.[1270]

The history of vaccination has largely been a willingness to trade some length of immunity—and maybe even a degree of robustness—in favor of avoiding the risk of an adverse outcome from infection. Most folk are sober-minded enough to recognize that in a pandemic almost everyone is going to be infected unless they either severely self-isolate or get vaccinated. Even with vaccination, while the odds of escaping improve, infection can occur. But because vaccination provides so much protection it lessens significantly the odds of having severe disease (including loss of life).

[1269] Quoted in Schittenhelm, "What Is Loss Aversion?" 72.

[1270] CDC Tracker (https://covid.cdc.gov/covid-data-tracker/#datatracker-home) as of May 27, 2022. The actual figure was 82.7% having received at least 1 dose.

Preparation Is the Best Prevention

Vaccines aim at infection prevention by teaching the immune system in advance to recognize and appropriately respond to attack by the agent being vaccinated against; as a secondary objective they aim to reduce the effects of any infection that does result, thus better protecting the person against the most serious effects of disease.

Vaccines constitute a critical primary way people can support the human immune system. The key to understanding how the immune system works is recognizing that it responds to *any* environmental agent it perceives as a threat, whether that agent is from nature or technology (which is why an immune response can be prompted by either COVID-19 or from a COVID-19 vaccine). Vaccines take advantage of this central truth of immune function and use it to prepare the system for encounters with environmental agents—like coronaviruses MERS, SARS-CoV, and SARS-CoV-2—that are far, far worse than any threat posed by the vaccine.

Metaphorically, an infectious agent such as a coronavirus is an enemy force expert at infiltration and rapid deployment. It quickly spreads its troops before an alarm is spread. By the time the host country—the human body—begins to muster its own troops (the immune system), significant damage may have occurred. Vaccines aim to prevent this by preparing the host defenses in advance. They recruit a readily armed force, teach it to recognize the enemy rapidly, and make possible a rapid deployment of defense forces should an invasion be attempted. Can soldiers be injured or died in training? Yes.—But preparedness saves far more lives than it costs.

The Logistics of Preparing for War

The philosophy of vaccines is preparing for a war one hopes never to have to fight. Pandemics, though, tell us that war is here and whether we like it or not we are at risk. No one gets to choose being a civilian when the homeland is invaded. All wars are costly, but preparing for one is a cost that saves money (and time) should war actually break out. *Vaccines are reckoned as the most cost-effective form of healthcare worldwide.* As pointed out a moment ago, vaccines prepare the body defense forces *before* the enemy invades. Most do this by helping the body prepare antibodies to recognize and effectively combat the enemy by—metaphorically—presenting a stand-in for the enemy, one who looks and acts like the enemy, but without his actual menace. But the truth is that developing an effective vaccine defense is not merely costly but faced by tremendous challenges. That is why scourges like malaria persist; no effective vaccine against them yet exists. Many pathogens, by virtue of complex biological cycles and/or success against immune system defenses, resist a line of defense that just aims at getting an antibody response ready. They require a more diverse immune response and that is a logistical challenge.[1271]

[1271] See Gray, Westerhoff, and MacLeod, "The Roles of Resident, Central, and Effector Memory CD4 T Cells," 574.

Three Important Functions of Vaccines

Vaccines have come to serve three important functions:

- First, *vaccines reduce the probability of infection* (and in a best case scenario entirely prevent infection).
- Second, *vaccines reduce the severity of symptoms*.
- Finally, *vaccines reduce the rate of transmission of disease*.

Obviously, two of these functions work at the level of the individual, while the third also works at the community level.[1272] The first two we have addressed throughout this volume, so we shall here focus on the third. The last function promotes *herd immunity*—an indirect benefit of enough people becoming immune or resistant to a disease so as to severely inhibit its spread. No one knows with certainty what percentage of a population needs to achieve immunity to reach herd immunity.

The third function—the *social benefit* of vaccination—has become a lightning rod in the debate over vaccines. That is because as vaccinations succeed individual risk of infection is lowered and some people 'piggyback' off the risk others have taken to enjoy the benefits of widespread immunization. Thus, relatively speaking, the private risk/benefit ratio is lower than the social risk/benefit ratio. Put another way, while it is clearly in the *public* interest to have widespread vaccination (low risk/high benefit), for any given *individual* the matter may be less clear and so motivation to get vaccinated may diminish.[1273] We actually have seen that with the COVID-19 pandemic. As more people became vaccinated and infection numbers dropped, fewer people chose vaccination—until infection numbers rose again. This is reflected in the so-called 'waves' of the pandemic. The more direct the threat is, the higher the motivation to act.

With respect to the social benefit of vaccines, as global health advocate Marianne Meijer and her colleagues put it, "In a pandemic, nobody is safe until everyone is safe." That means for vaccines to realize their full potential as a public health measure they need to be made available for everyone. In wealthy nations this is far less a problem than it is in poorer ones. As a result, people in the latter are often left to rely on a kind of *noblesse oblige* by other nations—a situation fraught with all the political and cultural complications that result whenever the rich and powerful flaunt that wealth and power—even in their giving. These considerations have considerably slowed progression toward coveted herd immunity. There are solutions, such as sharing intellectual property rights involved in vaccines, scaling up production of vaccines, and enacting other measures that enhance poorer nations' self-sufficiency and reliance. [1274]

[1272] Blume, *Immunization*, 19, points out that vaccines aim to facilitate two kinds of health—that of the individual body and the 'social body,' the community.

[1273] McDonnell and Toxvaerd, "How Does the Market for Vaccines Work?"

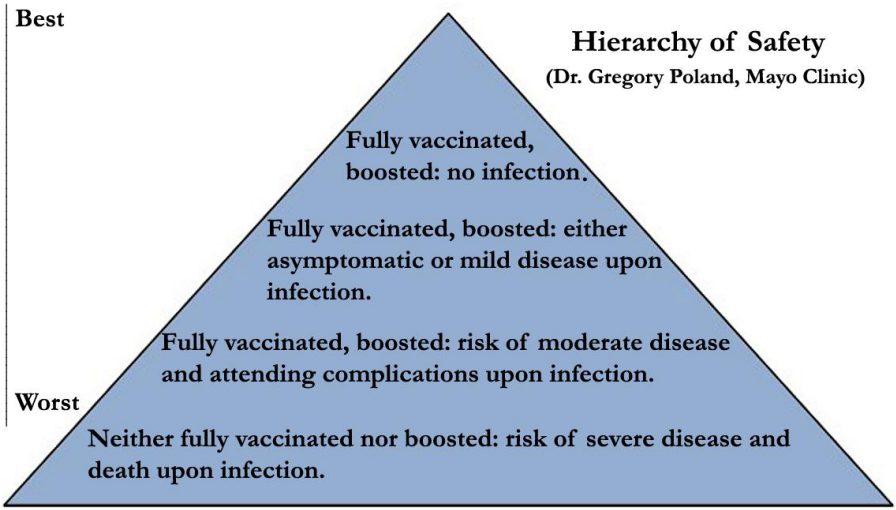
[1274] See Meijer, Vershuuren, and Weggen, "COVID-19 Vaccines a Global Good?" The quote is from p. 925.

Vaccines are judged not only by how well they work, but how safe they are. Numerous studies have consistently found strong effectiveness with reasonable safety for the approved vaccines.

Safety

Safety is a measure of how often and how severe adverse effects are following administration of the vaccine. These are typically measured at predetermined intervals. Thus researchers are interested in how a person responds immediately after administration of the vaccine, within the first week, within two weeks, and so on. The further out in time from the vaccine shots the less reliably can adverse effects be attached to the vaccine because so many more possible variables will have entered into the situation.

The degree of safety can be conceptualized. Director of the Mayo Clinic's Vaccine Research Group, Gregory Poland, offers a four point scale providing a 'Hierarchy of Safety.'



Poland also sounds a note of caution: "Best is getting three doses of an mRNA vaccine and never getting infected. Complications, including long COVID and transmission to others, represent a risk even in that scenario."[1275]

The issue of vaccine safety is one that in the United States has resulted in legal considerations stretching all the way to the Supreme Court. In 2010, in "Bruesewitz et al. v. Wyeth et al.," the Court in a 6-2 decision, affirmed a lower court decision that federal law shields vaccine manufacturers from certain product liability lawsuits in state court that seek damages for serious health problems suffered by children. Justice Scalia's opinion for the court's majority begins by

[1275] Poland quoted in Weise, "Full Vaccination," in which heirachy by Poland is also described.

acknowledging the success of vaccines and repeating the CDC judgment that they collectively constituted one of the greatest 20th century achievements of public health. Scalia goes on to observe that *their very success has left a public less worried about the diseases than the vaccines*. In this respect, the 1980s saw an increase in vaccine-related lawsuits. This had an adverse effect on vaccine makers, some of whom decided to discontinue their efforts. To address this situation Congress in 1986 created the National Childhood Vaccine Injury Act (NCVIA). The Act was designed to protect vaccine makers against spurious lawsuits while also protecting those with legitimate claims. In the case before the Court the vaccine in question was for DPT. Scalia parses the language of the NCVIA, then notes that the FDA has never specified criteria by which a vaccine is judged safe and effective for its intended use. He immediately adds, "And the decision is surely not an easy one. Drug manufacturers often could trade a little less efficacy for a little more safety, but the safest design is not always the best one. Striking the right balance between safety and efficacy is especially difficult with respect to vaccines, which affect public as well as individual health."[1276]

Efficacy

Efficacy (or *effectiveness*) is—technically—the proportionate reduction in disease in the vaccinated group. In real-world terms it is a measure of how well a vaccine does the following:

- prevent infection—the primary goal;
- prevent serious illness; and
- prevent death.

Because of an emphasis on prevention people are prone to judge harshly a vaccine that does not meet that goal—despite it being an unrealistic expectation that any vaccine should prove 100% effective at preventing infection. As seen in the history of vaccines only one has been so successful it eradicated the disease. But while 100% prevention of infection is unrealistic, very high levels of prevention of infection exist with vaccines, offering far better protection than simply taking one's chances against Nature.

Moreover, the other two goals also matter and are taken seriously. The reduction of *morbidly* rates (illness) and *mortality* rates (death) are weighty measures attesting to the effectiveness of vaccines. Though vaccinated people can still become infected, fall seriously ill, and die, they are far less likely to do so in any of these respects than are the unvaccinated.

Vaccine efficacy (VE) depends on a number of critical factors—and not all of them have to do simply with the vaccine itself. Opponents of vaccines often focus on vaccine production and design issues, or imperfect results, when there are far many more issues involved. It is to some of these we must now turn.

[1276] Supreme Court of the United States, "Syllabus: Bruesewitz et al. v. Wyeth et al." The quote is from Scalia's opinion, p. 13.

No less than is the case for the immune system, vaccine success depends on a number of critical factors. These broadly break into three categories as the following Table indicates.[1277]

Pathogen Factors	Host Factors	Vaccine Factors
antigenic mismatch	age	adjuvants
evasion/escape mechanisms	comorbidity (e.g., obesity)	administrative factors (e.g., site and route)
force of infection	immune history	dose
genetic variability	pregnancy	immunological memory
serotypes	sex	vaccine platform
transmissibility	targetable biological mechanisms	vaccination schedule

Because many of the factors listed have been discussed elsewhere in this book, here we will touch only on some that have not received adequate mention.

Antigenic mismatch occurs when the vaccine-induced antibodies do not match the pathogen strain of the infected recipient. This is something that has plagued annual flu vaccines,[1278] but it also is a concern with other mutating viruses.

A *pathogen's force of infection* (also referred to as 'hazard rate' or 'incidence rate') relates back to our earlier notion of susceptibility. Force of infection (FoI) refers to the rate at which susceptible individuals in a population during a specified period of time are infected by a pathogen circulating in that population. It has been argued that "for many but not all pathogens, modifying the FoI provides one of the most actionable interventions to enhance or sustain VE."[1279]

Earlier we met the idea of a *pathogen's serotype*—the distinctive surface presentation of antigens by a microbial entity such as bacteria or viruses. The number and nature of serotypes makes a difference. In viruses, serotypes generally correspond to genotypes. Antigen-specific antibodies that recognize one serotype typically are inefficient with respect to a different serotype of the same virus. This reality means that a vaccine meant to teach the body to produce antigen-specific antibodies to one serotype is handicapped if the virus has more than one serotype—a fact already established with at least one coronavirus and suspected with SARS-CoV-2.[1280]

[1277] A neat, but brief summary for all three areas is found in Falahi and Kenarkoohi, "Host Factors and Vaccine Efficacy," 1330. The host factors follow Dhakal and Klein, "Host Factors."
[1278] See, for example, Tricco et al., "Comparing Influenza Vaccine Efficacy."
[1279] Kaslow, "Force of Infection," 4.
[1280] Simon-Loriere and Schwartz, "Towards SARS-CoV-2 Serotypes?"

In earlier chapters, especially the one on the disease COVID-19, we examined a good number of *host factors*, including most of those listed above. In a manner parallel to differences in how individuals respond to a pathogen, so too there are differences in how they respond to vaccines. Similarly, it is a simple and basic reality that just as there are significant individual differences in immune response, vaccine efficacy varies among individuals. For example, antibody responses to yellow fever vaccine vary by more than 10 times, and for hepatitis B the vaccine variability between people can be more than 100 times.[1281]

Relatively long-lasting protection—'immunity'—depends on success at a *host's biological level*, which includes all of the factors mentioned above. The list, as virologist Santoch Dhakal and microbiologist Sabra Klein tell us, serves to remind that "the 'one size fits all' approach of vaccine administration does not necessarily protect equally across distinct ages, sexes, reproductive periods, or comorbid conditions, with commensal microbial, genetic, and hormonal mechanisms contributing to this variability."[1282]

Among issues being looked at today are *host targetable biological mechanisms*. Prominent among these is the role of *gut microbia* in vaccine response. Amidst the incredibly abundant symbiotic microbes found in the gut there are also abundant numbers of immune cells—an estimated 70% of immune cells are found in the digestive system. The interplay between gut microbia and immune cells constitutes a regulatory mechanism on the immune system. Because gut microbia and this interplay vary both among individuals and among populations around the world, there would seem to be an inevitable impact on vaccine efficacy. For example, differences in diet that create differences in gut microbia may help account for differences in vaccine response between people in rich and poor countries. It may be that manipulating microbiota (e.g., through nutritional interventions) can improve vaccine efficacy.[1283]

But at a cell level, one issue stands paramount. As scientists Anna-Karin Palm and Carole Henry put it, "The success of vaccines is dependent on the generation and maintenance of immunological memory."[1284] The phrase *immunological memory* refers to the body's ability to remember past dangers and respond upon recognition of their re-emergence. B-cells of the adaptive immune system provide what has been called 'two walls of protection'—*long-lived plasma cells* (LLPcs) *and memory B-cells* (MBCs).[1285] In response to a specific threat the adaptive immune system crafts a progressively more adept response in the form of antigen-specific antibodies. Just as the name suggests, 'antigen-*specific*' antibodies are designed to recognize and contest some important feature of the threat and thus contain or eliminate it.

[1281] Falahi and Kenarkoohi, "Host Factors and Vaccine Efficacy," 1330.
[1282] Dhakal and Klein, "Host Factors Impact Vaccine Efficacy," 10.
[1283] Falahi and Kenarkoohi, "Host Factors and Vaccine Efficacy," 1332–33.
[1284] Palm and Henry, "Remembrance of Things Past," 1.
[1285] Akkaya, Kwak, and Pierce, "B Cell Memory: Building Two Walls of Protection against Pathogens."

Infection elicits immunological memory, as does vaccination. The following illustration shows the two walls discussed above acting against a pathogen.

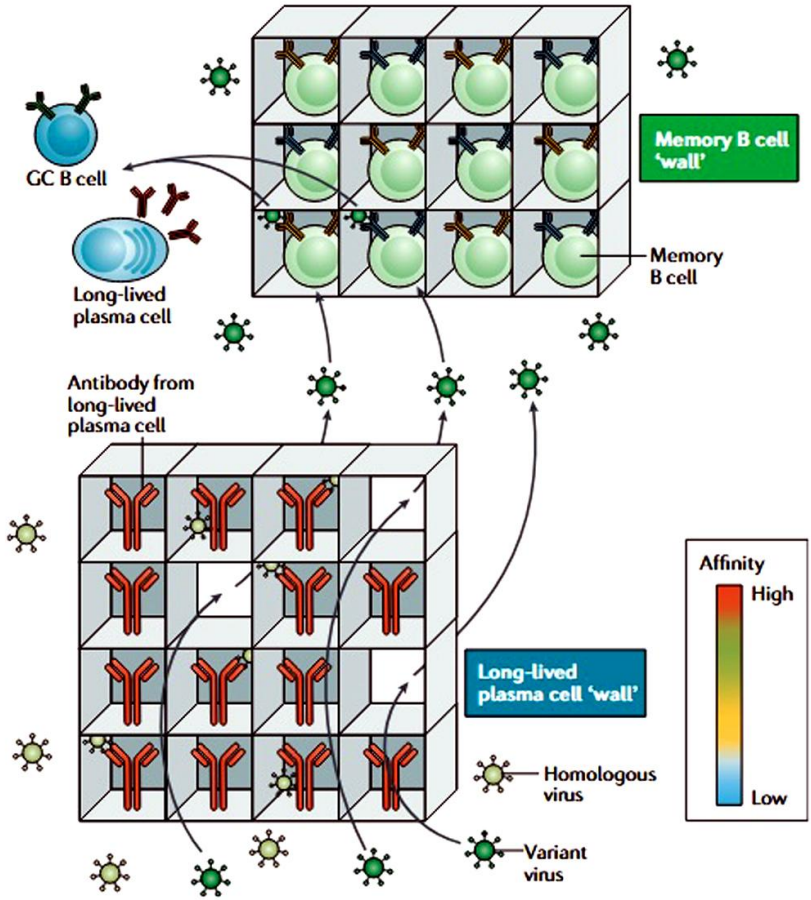


Fig. 4 | The role of long-lived plasma cells and memory B cells in the secondary response to antigen. Long-lived plasma cells in the bone marrow secrete highly selected, highly specific antibodies (depicted in red) that form a first 'wall' (bottom) against reinfection by homologous pathogens. Variant pathogens can find holes in this wall; however, having escaped the antibodies from the long-lived plasma cells, the variant pathogens encounter a second wall (top) formed by memory B cells that were less highly selected and therefore maintain a broader range of antigen affinities and specificities. The memory B cells are activated by the variant pathogen to differentiate into long-lived plasma cells or to re-enter the germinal centres (GCs) to replenish the memory B cell pool.

Illustration courtesy of Munir Akkaya, Kihyuck Kwak, and Susan K. Pierce, "B Cell Memory: Building Two Walls of Protection against Pathogens." *Nature Reviews Immunology* 20 (Apr., 2020) 229–38. https://doi.org/10.1038/s41577-019-0244-2. The illustration appears on page 236. As noted at article's end, this is a U.S. government work and not under copyright protection in the U.S.

As the Table above shows, *vaccine factors* include a wide range of things of different natures. Not all possible factors have been indicated in the Table for vaccines—or for pathogens and hosts. For example, there are factors affecting all three, such as geography, climate, level of nutrition, access to vaccines, and so forth.[1286] But the factors listed are all specific concerns that vaccine developers must consider.

Adjuvants are ingredients added alongside the key active ingredient with the aim of enhancing vaccine response. The Table below shows adjuvants in various vaccines used in the United States.

Adjuvant	Composition	Vaccines
Aluminum	One or more of the following: amorphous aluminum hydroxyphosphate sulfate (AAHS), aluminum hydroxide, aluminum phosphate, potassium aluminum sulfate (Alum)	Anthrax, DT, DTaP (Daptacel), DTaP (Infanrix), DTaP-IPV (Kinrix), DTaP-IPV (Quadracel), DTaP-HepB-IPV (Pediarix), DTaP –IPV/Hib (Pentacel), Hep A (Havrix), Hep A (Vaqta), Hep B (Engerix-B), Hep B (Recombivax), HepA/Hep B (Twinrix), HIB (PedvaxHIB), HPV (Gardasil 9), Japanese encephalitis (Ixiaro), MenB (Bexsero, Trumenba), Pneumococcal (Prevnar 13), Td (Tenivac), Td (Mass Biologics), Tdap (Adacel), Tdap (Boostrix)
AS04	Monophosphoryl lipid A (MPL) + aluminum salt	Cervarix
MF59	Oil in water emulsion composed of squalene	Fluad
$AS01_B$	Monophosphoryl lipid A (MPL) and QS-21, a natural compound extracted from the Chilean soapbark tree, combined in a liposomal formulation	Shingrix
CpG 1018	Cytosine phosphoguanine (CpG), a synthetic form of DNA that mimics bacterial and viral genetic material	Heplisav-B

The above Table excerpted from the Centers for Disease Control and Prevention (CDC) website page titled "Adjuvants and Vaccines" (https://www.cdc.gov/vaccinesafety/concerns/). This material is in the public domain. The final row of the CDC table, listing vaccines that do not use an adjuvant has been deleted.

Adjuvants, especially aluminum, have been a favorite target of anti-vaxxers. For example, they point out the inherent neurotoxicity of aluminum and remind us that some research finds toxic side-effects related to vaccination. In this regard, a 2015 paper authored by Gherardi et al. in *Frontiers in Neurology* is sometimes referenced. That paper found that aluminum adjuvants have a role in macrophagic myofasciitis (MMF) lesions. However, the same study refuted the belief *every* person vaccinated will experience MMF lesions. In the small number of adversely affected folk, MMF has been associated with chronic fatigue and other forms of myalgia (muscle pain). The researchers do *not* call for the elimi-

[1286] For a nicely illustrated overview showing the range of factors and their impact on vaccine efficacy, see Tregoning et al., "Progress of the COVID-19 Vaccine Effort," 629 (Figure 1).

nation of aluminum adjuvants but suggest further investigation to see "why a given individual will appear intolerant to alum-containing vaccines whereas the vast majority of individuals vaccinated with the same vaccines remain healthy."[1287] Aluminum (famously as the ingredient *alum* (aluminum oxy-hydroxide)) has been used in vaccines since the 1930s as an adjuvant. The exact mechanism(s) by which the desired effect is achieved remain uncertain—and none of the three approved COVID-19 vaccines include it—but research studies have shown that alum, *if* included in COVID-19 vaccines, would promote high concentrations of antibodies.[1288] Physiologist and well-known science educator Jonathan Berman points out that over 90 years of empirical evidence regarding alum's use in vaccines consistently demonstrates its safety. Moreover, the actual amount included is comparable to that found in infant formulas (and even natural breast milk has some aluminum).[1289]

Vaccine administrative factors may seem unlikely to impact vaccine efficacy, but they can. Such factors, and why they matter, include:

- *vaccination route*—for example, whether intramuscular (into a muscle) or subcutaneous (beneath the skin), can affect the dosage required to achieve the desired effect;
- *vaccination schedule*—spacing of doses matters as longer intervals between them often produces higher immune responses;
- *vaccination site*—where in the body the vaccine is received can sometimes affect responsiveness.[1290]

Vaccine dose is a factor that has received more attention in the current pandemic because of the extension of coverage from adults to children. As is true of other vaccines, less dosage is sufficient to achieve a comparable response; that often holds true for booster doses, too. But in some cases vaccine dosage needs to be increased to achieve the same desired result. This has been the case, for example, for some vaccines with pregnant women and elderly adults.[1291]

Vaccine platform—the 'type' of vaccine—is typically associated with injections—'shots'—though other means exist as well. We shall investigate this at further length later in this chapter with respect to COVID-19 vaccines. This matter will soon receive more extensive coverage with respect to COVID-19 vaccines. But we can start more broadly, first by listing kinds of platforms and then by drawing some observed general differences among selected types. The following two Tables will help us better understand what is going on with the development of COVID-19 vaccines.

[1287] Gherardi, et al., "Biopersistence." The quote is from p. 4. Also see Gherardi and Authier, "Macrophagic Myofascitis."
[1288] Hotez et al., "COVID-19 Vaccines," 399.
[1289] Berman, *Anti-Vaxxers*, 98–100 (quote is from p. 98).
[1290] This list selected from Zimmerman and Curtis, "Factors that Influence," 29–31, which see for extended coverage and examples.
[1291] See Zimmerman and Curtis, "Factors that Influence," 29.

The following Table shows the broad range of vaccine platforms.[1292]

Platform	Date of First Example of Type	Examples of Pathogens Vaccinated against by Platform
Live attenuated	1796	Measles, rubella, mumps
Killed whole organism	1896	Typhoid fever, hepatitis A
Nucleic acid (mRNA)	2020	COVID-19
Outer membrane vesicle	1987	Group B meningococcal
Protein-polysaccharide conjugate	1987	Pneumococcal (*Streptococcus pneumonia*)
Subunit	1970	Anthrax, pertussis
Toxoid	1923	Diphtheria, tetanus
Viral vectored	2019	Ebola
Virus-like particle	1986	Human papillomavirus

In addition to the above, there are also experimental vaccines (e.g., antigen-presenting cell; bacterial vectored) being explored.

The platform used makes a difference in various respects, including the number of doses required to achieve the desired effect. The following Table gives a brief account of this matter.

Vaccine Type[1293]	Doses typically Required	Notes
Live attenuated	1	Induces high vaccine response with long protection.
Inactivated	2+	Multiple doses (including boosters) may be required to reach efficacy of live vaccine.
Subunit	2+	Response may vary in duration and ages that can receive it.
Toxoid	2+	Multiple doses typically required.

[1292] Table based on Pollard and Bijker, "A Guide to Vaccinology," 87 (Figure 2).
[1293] Table based on Zimmerman and Curtis, "Factors that Influence," 27.

As one might expect by now, the above Tables hardly exhaust the matter. Historically and presently, a number of approaches have been and are being utilized for vaccines. The following list offers brief descriptions covering the ones listed above and more.

- *live-attenuated virus vaccines* (LAV)—those that use the live virus, but in a seriously weakened state;
- *inactivated virus vaccines* (IVV)—those that use the dead virus;
- *subunit, polysaccharide, conjugate, and toxoid vaccines*—those that target some specific thing associated with the virus;
- *recombinant vaccines*—those that use laboratory technology to combine parts;
- *viral vector vaccines*—those that use a harmless virus (the 'vector') to carry information to prepare the immune system;
- *plant-based vaccines*—these utilize non-infectious protein particles that mimic viral structures to prompt a human immune response;
- *erythrocyte-based virus-like particles* (Erythro-VLPs)—these use red blood cells (*erythrocytes*) as endogenous carriers of the spike (S) protein;
- *DNA vaccines*—those that use a DNA sequence to generate an immunogenic protein from the person vaccinated; and,
- *mRNA vaccines*—those that make special messenger RNA molecules to instruct an immune system response.

All of these vaccine types include provoking a mild immune response indicating a response by the immune system in preparation against actual viral infection. *Please Note*: None of the approved vaccines for COVID-19 use in the United States include the actual SARS-CoV-2 virus, either dead or alive.

The kinds of vaccines associated with COVID-19 will be elaborated later, but some further brief remarks to elaborate on the material of the last couple of pages seem appropriate here. The first two listed above—live-attenuated and inactivated—are both often grouped under the category *whole virus* vaccines. The subunit, polysaccharide, and conjugate vaccines all can be quickly described. The *subunit* vaccine targets a specific protein of the virus, especially one vital for attachment or replication. A *polysaccharide* vaccine targets the sugary molecules (polysaccharides) that are vital to cell structure and function. The vaccine stimulates B-cell response of the immune system. This enhances the work of immune system cells called phagocytes (see previous chapter). *Conjugate* vaccines also have polysaccharides in mind—those of bacteria. They 'conjugate' ('couple') together polysaccharides (typically from the surface coat of the bacteria) with protein carriers and, as in polysaccharide vaccines, stimulate an immune system response through this joining of a weak and a strong antigen. *Toxoid* vaccines target some toxin ('poison') produced by the infectious agent. These vaccines first render a harmless form of the toxin, then inject it to provoke an immune system defense built against future exposure to the real thing. Both DNA and

mRNA vaccines are sometimes grouped as *nucleic acid vaccines* because both DNA and RNA refer to nucleic acids.

Recombinant vaccines are of a variety of kinds. The name 'recombinant' means 'formed by recombination' and that entails technology. A *recombinant vector vaccine* engineers a 'vector'—a modified virus or bacteria—to deliver genetic code for an antigen. They do this with extra genes derived from the live agent the vaccine is designed against. Recombinant and *DNA* vaccines can be similar but the difference is a notable one. A recombinant vaccine doesn't actually use DNA but employs technology to synthesize protein or glycoprotein subunits; a DNA vaccine actually introduces into the vaccinated person a DNA sequence encoding the antigen to which an immune response is desired. It prompts both B-cell and T-cell responses by the immune system.

Vaccine-Induced Antibodies

Regardless of platform, vaccines typically aim at encouraging production of vaccine-induced antibodies. Broadly speaking, these are one or the other—or both—of the two kinds of antibodies we have previously discussed:

- *neutralizing antibodies* (nAbs)—antibodies that bind to specific antigens, typically to block attachment and penetration by the virus; and.
- *non-neutralizing antibodies* (n-nAbs)—antibodies using various mechanisms in anti-viral activities.

Vaccines can induce both kinds; the yearly influenza shots exemplify this outcome.[1294] As a general rule, vaccines desire to produce neutralizing antibodies.

Neutralizing antibodies tend to get the most press. They are the frontline vaccine defense, meant to stop infection. These target surface proteins on a virus, such as the envelope (E) protein, or in the case of coronaviruses, the spike (S) protein. However, these proteins are the very ones on a virus most likely to mutate. The less 'conserved'—that is, 'stable'—a viral genome is, the more likely it will be that an adjusted vaccine (e.g., a booster shot) will be needed. Vaccines can thus come to resemble a game of Whac-a-Mole—a series of efforts to whack the 'mole' of the virus as a new form appears. But no one wants an endless string of vaccine shots.

Non-neutralizing antibodies might be called more subtle—and mysterious. At this point their functional significance against enveloped viruses—which include the coronaviruses—remains poorly understood.[1295] They do not prevent infection—which is why they are called 'non-neutralizing'—but can still contribute significantly to fighting viruses. They target the more conserved parts of a virus, proteins like the nucleoprotein of influenza viruses. If their function can be in-

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[1294] Henry Dunand et al., "Both Neutralizing and Non-Neutralizing."

[1295] Earnest et al., "The Mechanistic Basis of Protection by Non-Neutralizing Anti-Alphavirus Antibodies," 1.

terrupted and immunity thus developed, then a measure of protection is afforded against multiple variants and their subtypes.[1296]

Another kind of 'non-neutralizing antibody' is an antibody intended to be neutralizing but that fails in that respect. It is functionally 'non-neutralizing.' The danger of that, as we have seen, is antibody-dependent enhancement (ADE)—an unintended outcome that vaccine-makers must be vigilant against.[1297]

Obstacles to Vaccines

Three notable obstacles exist for most vaccines:

- creating a safe and effective vaccine;
- overcoming fear of shots; and,
- addressing vaccine hesitancy and opposition.

The first of these is the only one inherently necessary. The other two are much more problematic.

Obstacle 1: Creating a Safe and Effective Vaccine

Because this matter is covered more completely in the following chapter, here the remarks will confine themselves to the perception of adverse events.

Adverse Events

Adverse Events (AEs) are those events subsequent to vaccination in which someone—typically the person vaccinated, but perhaps also an observor—reports some negative ('adverse') happening associated with the vaccine.

It would be irresponsible not to acknowledge that someone can die of a vaccine. But it would be just as irresponsible to suggest that the danger of dying from a vaccine is anywhere near as likely as dying from a pathogen like COVID-19. The possibility from the latter is so much greater that anti-vax suggestions to the contrary are the worst example of deceitful fear-mongering.

Yet people do die, and the remotest possibility of it is one reason some hesitate to be vaccinated. Faced with what they see as 'the Devil (vaccine) or the Deep Blue Sea (COVID-19)' they may choose navigating the sea in hopes of somehow finding a safe voyage. But this can look a lot like choosing a short-term 'sure thing'—no risk of adverse reaction to a vaccine—despite the far higher odds of infection absent vaccine protection followed by the far higher odds of adverse symptoms. This rather resembles people with neuropsychiatric disorders such as addiction or pathological gambling, who often choose pursuing short-term gain even at the cost of severe long-term adverse outcomes.[1298]

[1296] See Carragher et al., "A Novel Role for Non-Neutralizing Antibodies." Also see Sedova et al., "Non-Neutralizing Antibodies Directed at Conservative Influenza Antigens."
[1297] See Iwasaki and Yang, "The Potential Danger of Suboptimal Antibody Responses," 340.
[1298] Bechara, "Neurobiology of Decision-Making: Risk and Reward."

Of course, the latter may be an extreme, but the underlying risk-taking is not outrageously different.

However, it is educational to examine reported deaths. For example, one reported in early 2022, during the Omicron wave, was of a 68 year old woman who died of a severe allergic reaction (*anaphylaxis*) to the vaccine. However, in addition to her age, she had a previous history of allergic reactions to drugs and underlying health conditions (high blood pressure (*hypertension*), reactive airway disease, etc.).[1299] The CDC has estimated an extreme allergic reaction occurs in two to five people per million.[1300] In fact, an examination of claims by a prominent anti-vaxxer about deaths due to the vaccine found that a large number of the death entries noted the deceased was elderly and/or infirm, and many of the entries submitted by actual medical clinicians presumed the recorded death was *not* related to the vaccine. That same examination points out that many factors are observed in cases, such as the person committing suicide or already having been infected when vaccination was begun. In one case, for example, the deceased was 99 years old, had refused all food for a week, and died within 12 hours of receiving the vaccine.[1301]

Adverse events can be reported to the *Vaccine Adverse Event Reporting System* (VAERS). This publicly accessible system provides a U.S. database for reporting alleged side-effects. Reports can be submitted by anyone. The key in understanding its database is keeping in mind that all reported events are *alleged*—not proven—side-effects. The reports are *unvetted*, meaning they have not been verified and are by their nature unreliable sources in any scientific sense. The VAERS database itself explicitly declares, "The reports may contain information that is incomplete, inaccurate, coincidental, or unverifiable. In large part, reports to VAERS are voluntary, which means they are subject to biases."[1302]

The VAERS system by its very nature is malleable; it can be manipulated. Some evidence suggests that it is used by anti-vax advocates to spread misinformation by selective reporting of numbers taken out of context, using select reports as 'testimonials' of vaccine danger, or even by creating false reports.[1303] An example of the kind of thing that can happen is when one person is alleged to have filed a report saying the vaccine turned him into the Incredible Hulk.

A substantial percentage of adverse events are likely not legitimately attributed to the vaccine. In early 2022, a systematic review and meta-analysis of 12 studies, with AE reports from 45,380 people (22,578 (49.7%) of whom had received a placebo), examined both *local adverse events* (i.e., those occurring at the site of injection), and *systemic adverse events* (i.e., those occurring in places distant from the shot) for both those who received the vaccine and those who did not. This study reported that among placebo groups about one-third of the partici-

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[1299] Fink, "Woman Died of Allergic Reaction to COVID Vaccine."
[1300] CDC, "Selected Adverse Events."
[1301] Weir, "How Robert F. Kennedy Jr. Became the Anti-Vaxxer Icon."
[1302] VAERS, "Disclaimer" (https://vaers.hhs.gov/data.html.).
[1303] For more on this matter, see Brumfiel, "Anti-Vaccine Activists."

pants reported an AE. Of reports from those who did receive shots, more than three-quarters (76%) of systemic AEs and about a quarter (24.3%) of local AEs after the first shot in a two shot series were attributable to the *nocebo effect*—a symptom explained best by *the patient's expectation* that the effect will occur because of the vaccine. After the second shot the nocebo effect diminished but was still significant—over half (51.8%) of systemic AEs and more than a sixth (16.2%) of local AEs could be attributed to the nocebo effect. Such common nonspecific complaints as having a headache or experiencing fatigue—found in both vaccinated and placebo groups—were particularly associated with the nocebo effect.[1304] Accordingly, simple numbers of reported AEs do not tell the whole story.

Obstacle 2: Overcoming a Fear of Shots

Some of the events reported to VAERS may be related to the *experience* of getting vaccinated (the setting, or fear of needles), rather than the actual vaccine. For instance, are events like anxiety, a cold sweat, dry mouth, abnormal sweating, an elevated heart rate, palpitations, or even loss of consciousness (e.g., from fainting)—all sometimes reported to VAERS—more likely attributable to the vaccine experience or the vaccine itself?

Many people have a fear of needles and injections—of getting *shots*. Though the technical name assigned is *trypanophobia*, the affliction is more commonly called *needle phobia*. Symptoms can include some or all of the following:

- anger or loss of control;
- anxiety, including an anxiety attack;
- breathlessness;
- dry mouth;
- fear of dying;
- feeling sick;
- heart palpitations;
- inability to think clearly;
- sensation of detachment from reality; and/or
- trembling.[1305]

Courtesy of FDA

Professor of clinical psychiatry and pediatrics at UCLA's David Geffen School of Medicine, Brenda Bursch, observed in an interview that the stigma associated with phobias is one reason the topic is rarely discussed. "If you are somebody who otherwise prides themselves on being stoic or being able to

[1304] Haas et al., "Frequency of Adverse Events." See especially p. 11.
[1305] Raghvendra et al., "Tryphanophobia," 18.

handle stress or a high-pressure job, needle anxiety could feel incongruous to your personality. So, I think it's not something people talk about frequently."[1306] That reality makes it likely instances of a fear of needles are under-reported.

Some studies have examined this fear with respect to COVID-19. For example, an article published in June, 2021, reported on research with more than 15,000 adults in the United Kingdom. This group was sampled to match the population for age, gender, ethnicity, income and region. More than a quarter (26.2%; N=3,927) screened positive for 'blood-injection injury phobia.' These individuals were about twice as likely to express hesitancy to COVID-19 vaccination as others (22% to 11.5%).[1307] In the U.S., a study reporting on 804 English-speaking adults found that 11.8% of them admitted that a 'hate' of needles and injections contributed to their hesitance to COVID-19 vaccination.[1308]

Obstacle 3: Addressing Vaccine Hesitancy and Opposition

Who are the vaccine hesitant? The following Table offers some demographics obtained from 713 individuals participating in an online survey

TABLE 1 | Demographics of online survey panel, United States, March 2020.

		Count (column %)	Vaccine hesitant (row %)	Reject COVID-19 vaccine (row %)
Overall		713 (100%)	230 (33.0%)	131 (18.4%)
Participant's gender	Male	326 (45.7%)	98 (31.0%)	51 (15.6%)
	Female	387 (54.3%)	132 (34.8%)	80 (20.7%)
Participant's residence	Rural	227 (32.5%)	88 (40.2%)	37 (16.3%)
	Urban	471 (67.5%)	139 (29.9%)	93 (19.7%)
Participant's generation	Baby boomer and silent generation	242 (34.1%)	48 (20.5%)	40 (16.5%)
	GenX	222 (31.3%)	60 (27.6%)	41 (18.5%)
	Millennial	176 (24.8%)	80 (46.2%)	32 (18.2%)
	GenZ	70 (9.9%)	41 (59.4%)	17 (24.3%)
Participant's race/ethnicity	Non-Hispanic White	531 (74.5%)	146 (28.0%)	86 (16.2%)
	Non-Hispanic Black	50 (7.0%)	33 (70.2%)	17 (34.0%)
	Hispanic	53 (7.4%)	24 (47.1%)	12 (22.6%)
	Other	79 (11.1%)	27 (36.0%)	16 (20.3%)
Monthly family income	<$2,000	140 (20.2%)	70 (51.1%)	39 (27.9%)
	$2,000-$4,999	198 (28.5%)	70 (36.3%)	43 (21.7%)
	$5,000-$9,999	212 (30.5%)	60 (28.7%)	30 (14.2%)
	≥$10,000	144 (20.7%)	27 (19.1%)	18 (12.5%)
Political affiliation	Republican	216 (31.8%)	71 (33.3%)	37 (17.1%)
	Democrat	262 (38.5%)	76 (29.7%)	41 (15.6%)
	Independent	202 (29.7%)	74 (37.6%)	51 (25.2%)
Perceived risk of infection within next month	*median (IQR)*	*32% (11%-51%)*	–	–

Table from Shih, Shu-Fang, et al. "Vaccine Hesitancy and Rejection of a Vaccine for the Novel Coronavirus in the United States." *Frontiers in Immunology* 12 (June, 2021) art. 558270. doi: 10.3389/fimmu.2021.558270. Material appears on page 4 and is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/ legalcode). The original has been changed in font size and type for explanatory material.

[1306] Quoted in Karmarker, "Treating Needle Anxiety Can Boost COVID-19 Vaccination Rates."
[1307] Freeman et al., "Injection Fears and COVID-19 Hesitancy." See especially p. 9.
[1308] Ruiz and Bell, "Predictors of Intention to Vaccinate against COVID-19," 1083 (Table 3). The percentages by group were: 'extremely unlikely' (8.2%); 'somewhat unlikely' (17.4%); 'unsure' (11.9%).

There are many variables that contribute to vaccine hesitancy and/or vaccine opposition. Let's start with an overview of some *individual psychosocial factors*.[1309]

Individual Psychosocial Factors in Vaccine Hesitancy	
Beliefs and convictions about health and/or immunity (e.g., 'natural immunity is better than artificial means')	*Perceptions of vaccination's importance to health* (e.g., a belief that vaccine preventable diseases (VPDs) are important to building a strong immune system or that good personal hygiene is sufficient)
Health professionals' advocacy of alternatives (CAM=complementary and alternative medicine, which are often advanced by chiropractors and naturopaths)	*Religious beliefs/convictions* (e.g., that seeking vaccination reflects a lack of trust in God's providence, or that vaccines use illicit materials such as cells from aborted fetuses)
Knowledge/information about vaccination	*Sense of social responsibility* (low sense of public duty to work toward herd immunity or to protect others)
Past experiences (e.g., with health professionals, but especially with vaccination services)	*Subjective norm* (the influence of respected others) can lead to *Social pressure* for or against vaccination
Perceptions of risk (e.g., estimation of personal risk of infection and possible risk of vaccine side-effects)	*Trust* in health professionals and others (e.g., public officials, government)

Some of the above have been adequately addressed elsewhere in this volume (and in my *Understanding COVID-19 Vaccination Arguments*), but to others can be added some further comments below as well as some notice of other factors.

Beliefs at the individual level or among groups of people (e.g., religiously-affiliated or politically-affiliated groups) can contribute to vaccine hesitancy. Often these are shrouded in an apologetic rationale such as, 'I have done my homework and have good reasons for my hesitancy'—thus protecting their belief system by appealing to reason and evidence whether or not such have actually had much role.

[1309] The Table is based on Dubé et al., "Vaccine Hesitancy: An Overview," 1768–70; also see their comments on other possible contributors to hesitancy, such as the role of media and communication, public health and vaccine policies, and the role of health professionals (1765–67). Also see Bolich, *Understanding COVID-19 Vaccination Arguments*, 7, 22–26.

Let's compare a couple of published studies that examine the kinds of beliefs or reasons offered for vaccine hesitancy/refusal with respect to COVID-19.

Study 1 (*nationwide survey in the United States*)[1310]	**Study 2** (*review of 15 studies*)[1311]
[1] the possibility of dangerous vaccine side-effects	[1] general opposition to vaccines
[2] the possibility of an allergic reaction	[2] concerns about safety (e.g., not enough time spent developing it)
[3] the possibility the vaccine wouldn't work	[3] unnecessary because COVID-19 is harmless
[4] preference for 'natural' immunity	[4] general lack of trust
[5] belief in personal low-risk for infection	[5] doubts about vaccine effectiveness

The danger of beliefs is they all too often substitute for actual knowledge. Thus, with hesitancy to measles vaccination in mind, sociologists Julie Brownlie and Alexandra Howson have observed what they term the 'leap of faith' parents make when, having critical knowledge gaps about the vaccine (e.g., the fact that the study that undermined confidence was later proved the fabricated work of someone who was developing an alternative vaccine), they make a decision for or against vaccination. Such parents move beyond trust from a base in knowledge to one based in emotional interactions and familiarity with health professionals.[1312] In short, personal trust—or lack of it—becomes a critical factor.

Confidence and *trust* are general factors that experts agree are important, too. These factors are especially prominent with respect to people's attitudes about science, physicians and other public healthcare providers. However, matters of confidence and trust extend also to other public figures like politicians and religious leaders, many of whom have weighed in on the matter of vaccination. These factors relate, too, to attitudes about the World Health Organization (WHO), national health ministries such as the CDC, and local health departments. Such issues were examined early in the pandemic via an online survey in seventeen countries/territories across five world regions between May–June, 2020. The researchers found that the strongest predictors of vaccine hesitancy were confidence in the WHO and trust in health practitioners. However, levels of confidence and trust in scientists, local health departments, and national health ministries were also predictive, as were such levels with respect to reli-

[1310] Ruiz and Bell, "Predictors of Intention to Vaccinate against COVID-19," 1083 (Table 3).
[1311] Troiano and Nardi, "Vaccine Hesitancy in the Era of COVID-19," 250.
[1312] Brownlie and Howson, "'Leap of Faith' and MMR: An Empirical Study of Trust."

gious leaders. On the other hand, trust and confidence in political leaders was not predictive.[1313]

As vaccination for COVID-19 extended to cover children and adolescents there emerged forcefully the already lurking problem of *parental vaccine hesitancy (or refusal)*.[1314] Such hesitancy has been seen with regard to other vaccines and is, in part, a reflection of the fact that most vaccinations are recommended in childhood and at age when young parents are anxious to avoid risks.

Personality has a bearing on behavior and the choices people make, including whether or not to be vaccinated. "Vaccine hesitancy is a multidimensional concept," researcher Matt Howard explained in an interview with *PsyPost*, "and beliefs that vaccines have significant health risks as well as beliefs that someone is too healthy to need vaccines are two dimensions with a particularly strong effect on the decision to become vaccinated."[1315]

Howard, in a study published early in 2022 in the journal *Personality and Individual Differences*, examined this subject using three frameworks of orientation to the world—one positive, one negative, and one neutral. All are useful for understanding relatively stable individual differences. Perhaps the most famous of these frameworks is the neutral one, the 'Big Five' or 'Five Factor' model, often remembered by students by the acronym OCEAN (**O**penness, **C**onscientiousness, **E**xtraversion, **A**greeableness, and **N**euroticism). The second framework is called 'The Dark Triad' (Machiavellianism, Narcissism, and Psychopathy)—three maladaptive orientations. The third is a positive framework termed 'Psychological Capital' (Self-efficacy, Hope, Optimism, and Resilience). Alongside these frameworks Howard added eight measures related to Vaccine Hesitancy (Health Risk, Cost, Physical Pain, Inconvenience, Personal Reactions, Access, Healthiness, and Forgetting).[1316]

The study found that Psychological Capital was significantly related to a willingness to be vaccinated and that extraversion also was positively related. With respect to Vaccine Hesitancy, two factors—the aforementioned belief that vaccines pose a health risk and the belief that the person is too healthy to need vaccination—emerged as most prominent. These beliefs appear to correlate with certain personality dispositions. Those more likely to be vaccine hesitant, or oppose vaccination, had stronger associations with narcissism and psychopathy (i.e., a tendency to be selfish, antisocial, and impulsive). Surprisingly, the trait of conscientiousness—a tendency toward duty and detail—was also correlated with a reluctance to be vaccinated. Howard suggests this may be because this trait has been shown to be associated with overconfidence—a belief that their efforts can overcome obstacles and that what they are already doing is enough to compensate for a lack of vaccination.[1317]

[1313] Rozek et al., "Understanding Vaccine Hesitancy."
[1314] On the general matter, see Olusanya et al., "Addressing Parental Vaccine Hesitancy."
[1315] Howard quoted in *PsyPost*, "New Research Uncovers a Surprising Link."
[1316] Howard, "The Good, the Bad, and the Neutral," 1–2.
[1317] Howard, "The Good, the Bad, and the Neutral," 3.

Coronavirus Vaccines

By the summer of 2021, over 230 different COVID-19 vaccines were under development around the world. These were a diverse lot, including plant-based vaccines[1318] and nasal spray vaccines.[1319] By the end of 2021, the World Health Organization (WHO) was reporting 194 pre-clinical vaccines in development, with 23 presently in use around the world.[1320] Outside of the United States, where only three have been authorized for use, probably the one most recognized by Americans is the AstraZeneca *Vaxzevria* vaccine used in Europe (a recombinant adenovirus vaccine). AstraZeneca by the end of 2021 had seen administration in more than 180 countries.[1321] China's *CoronaVac* was one of the mostly widely used vaccines in the world, as was *Covilo*.

Within the United States, three vaccines were authorized for use: Moderna's, Pfizer/BioNTech's and Johnson & Johnson's. As of August 31, 2021, most of the 61% of vaccinated Americans had received either Pfizer's (53%) or Moderna's (39%) mRNA vaccine. By the end of the first week of June, 2022, the same was true for most of the 77.9% who had received at least one dose (Pfizer, 60.9%; Moderna, 39%; Johnson & Johnson, 0.03%).[1322]

Some Major COVID-19 Vaccines

Common Name	*Designation*	*Mechanism of Action*
AstraZeneca *Vaxzevria*	ChAdOx-nCov19 (ChAd; AZD1222)	Replication-deficient adenovirus vectored vaccine expressing S protein
Convidicea	(PakVac; Ad5-nCov)	Recombinant (adenovirus vector)
Covaxin	BBV152	Inactivated virus vaccine
Johnson & Johnson (Janssen)	Ad26.COV2.S (*JNJ*-78436735)	Replication-deficient adenovirus vectored vaccine to encode full S protein
Moderna	mRNA1273 (m1273)	mRNA vaccine encoding S protein
Novavax	NVX-CoV2373 (NVX)	Nanoparticle vaccine containing purified S protein
Pfizer/BioNTech	BNT162b2 (BNT)	mRNA vaccine encoding S protein
Sanofi-GSK	VAT00002, VAT00008	Adjutavant Recombinant Protein-based using S protein
Sinovac Biotech Ltd *CoronaVac*	DB15806 (PiCoVacc)	β-propiolactone-inactivated virus vaccine with aluminum hydroxide adjuvant
Valneva	VLA2001 (VLA)	Whole, inactivated SARS-CoV-2 virus
Vero Cells/*Covilo*	BBIBP-CorV	Inactivated SARS-CoV-2 virus

[1318] Dress, "Plant-Based Coronavirus Vaccine." Through phase 3 trials the GlaxoSmithKline vaccine showed 71% vaccine effectiveness against all variants but Omicron.

[1319] Rubin, "COVID-19 Nasal Spray," discusses the development of this application to the already existing AstraZeneca vaccine. Five such sprays had reached clinical testing stages by June, 2021.

[1320] Gavi, "The COVID-19 Vaccine Race."

[1321] Munro et al., "Safety and Immunogenicity of Seven COVID-19 Vaccines," 17.

[1322] For 2021, Bruxvoort et al., "Real-World Effectiveness of the mRNA-1273 Vaccine," 2. For 2022, CDC, "COVID Data Tracker" (June 6, 2022; https://covid.cdc.gov/covid-data-tracker/#vaccinations_vacc-total-admin-rate-total.)

Basic strategies for COVID-19 vaccines can be illustrated thusly:

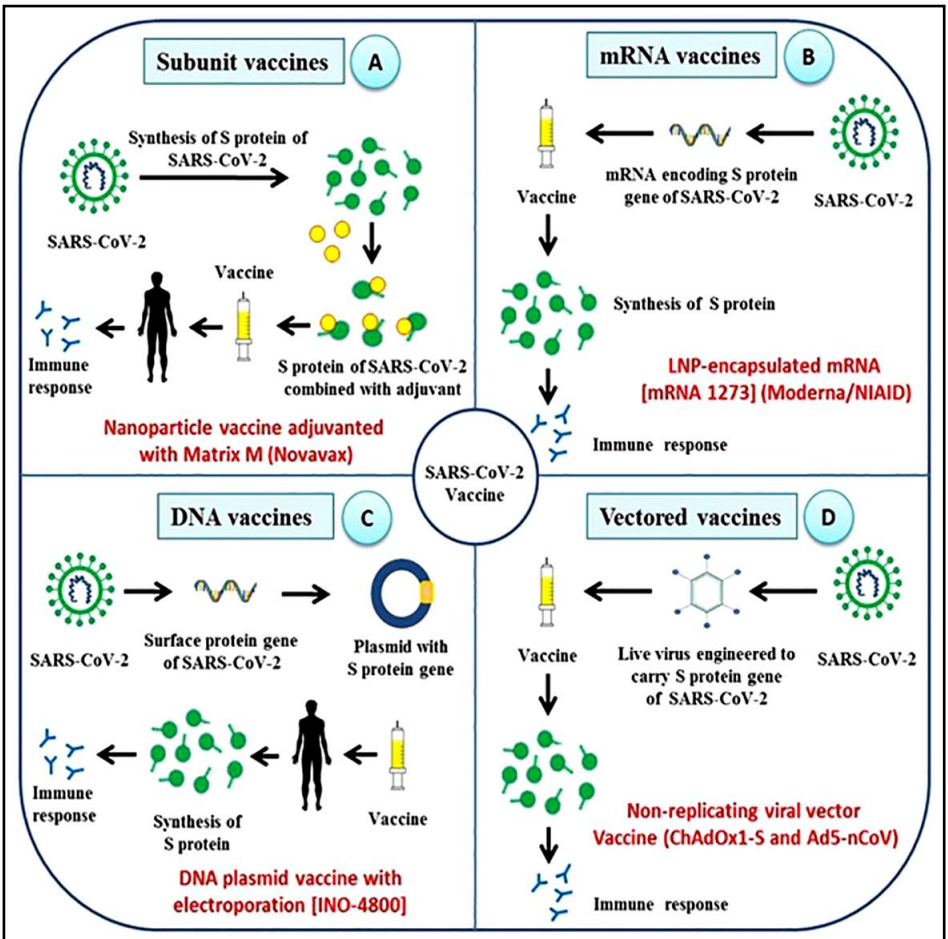

FIGURE 3 | Different strategies employed for the development of vaccines against SARS-CoV-2. Research institutions along with pharmaceutical companies across the world are adopting unique strategies to develop effective vaccines against SARS-CoV-2 and most of them target spike proteins and its functional domains. (A–D) The type of vaccine being developed includes subunit vaccine, mRNA vaccine, DNA vaccine and viral-vector based vaccines which aim to elicit humoral as ell as cellular immune response against spike protein of SARS-CoV-2.

Illustration is from Yashpal S. Malik, Prashant Kumar, Mohd Ikram Ansari1 Maged G. Hemid, et al. "SARS-CoV2 Spike Protein Extrapolation for COVID Diagnosis and Vaccine Development." *Frontiers in Molecular Biosciences* 8 (July, 2021) art. 607886. doi: 10.3389/fmlob.2021.607886. Material appears on page 7 and is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/legalcode). The original has been changed in font size and type for explanatory material.

At the time this book was being finished the work of developing vaccines against COVID-19 was far from done. One promising line of pursuit involves *protein-based vaccines*. These use purified virus protein plus adjuvants—agents used to enhance a vaccine's effectiveness. The Shingrix vaccine for shingles is an example of this kind of vaccine approach. Two such vaccines designed for COVID-19 (Novavax and Sanofi/GSK) use cells from *Spodoptera frugiperda* (the fall armyworm) to synthesize the protein.[1323]Another uses the SARS-CoV-2 Spike (S) protein's receptor-binding domain (RBD) fused to an alpaca-derived nanobody. Though it has taken longer to develop than the mRNA vaccines it is touted as easy to manufacture and—importantly—robust against variants.[1324]

COVID-19 Vaccines: Nasal Spray Delivery Vaccine Candidates

It should be noted that not only are vaccines different in type along the lines already indicated, but also in terms of *delivery system*. Almost all vaccines are delivered by *shots*, but some are available also by *nasal spray*. With respect to vaccinating against COVID-19, by the spring of 2021, only a few vaccines utilizing nasal spray delivery had reached the stage of clinical trials. Among those in various stages of development are the following:

Vaccine	Country (Company)	Mechanism of Action
603392.SS	China (Beijing Wantai)	weakened flu viruses such as H1N1, H3N2 and B with genetic segments of COVID-19's Spike (S) protein[1325]
Mambisa (CIGB-669)	Cuba (Center for Genetic Engineering and Biotechnology (CIGB))	recombinantly produced antigens[1326]
FinnCoVac	Finland (Rokote Labs)	Replication-deficient adenovirus-vector vaccine[1327]
BBV154	India (Bharat Biotech)	chimpanzee adenovirus-vector vaccine[1328]
COVI-VAC	UK (Codagenic)	live attenuated virus vaccine[1329]
AdCOVID	U.S. (Altimmune)	adenovirus-vector vaccine[1330]
AuNS-Chitosan	U.S. (Stanford Univ.)	DNA vaccine expressing SC2 S Protein[1331]

[1323] Dolgin, "How Protein-Based COVID Vaccines Could Change the Pandemic."
[1324] Pishesha et al., "A Class-II MHC-Targeted Vaccine." See esp. p. 6.
[1325] Reuters, "Beijing Wantai Plans Large Trial."
[1326] Rodriguez et al., "Mambisa."
[1327] Teivainen, "Finnish COVID-19 Vaccine."
[1328] Unsigned, "Bharat Biotech Seeks Nod for Phase-3 Trials."
[1329] Arthur, "Needle-Free COVID-19 Vaccine."
[1330] Altimmune, "Altimmune Commences Enrollment in Phase 1 Clinical Trial."
[1331] Kumar et al., "Gold-Nanostar-Chitosan-Mediated Delivery of SARS-CoV-2 DNA Vaccine."

In addition to these, there are also efforts to adapt approved vaccines like that from AstraZeneca for nasal spray delivery.[1332] As of early May, 2022, three nasal spray vaccines were in stage 3 (human) trials with large groups. These were attended by twin hopes: that the means of administration would increase the number of those choosing vaccination, and that they might trigger stronger immunity while offering protection against SARS-CoV-2 variants.[1333]

COVID-19 Vaccines: Red Blood Cell (RBC) Delivery Vaccine Candidate

In early 2022, a team of immunologists, chemists, and physicists at McMaster University in Canada announced research efforts to develop a red blood cell (RBC) method of vaccine delivery. Formally termed an approach using *erythrocyte-based virus-like particles* (Erythro-VLPs), the vaccine employs RBCs as an endogenous—'natural'—delivery method. The goal remains a familiar one—to deliver the spike (S) protein of SARS-CoV-2 to human host cells in order to prompt the immune system to recognize it and respond effectively.[1334]

The idea itself has been around awhile. The attractiveness of using RBCs as a delivery method stems from their natural safety as a carrier and their abundance—a single microliter of blood contains some 5 million RBCs and the total number in the body approaches about 30 trillion. Moreover, they have a lifespan of 100–120 days (3–4 months), and are ideally sized molecules for carrying various therapeutic agents. But though a wide range of applications for this delivery method have been studied, there are challenges this approach must overcome, such as safely matching blood types and avoiding blood-born infections. Still, these are manageable issues that have been effectively addressed.[1335]

The Canadian team's approach is to create Erythro-VLPs—S proteins anchored in the cell membrane (the cytoplasmic membrane) of RBC liposomes, a cell 'package' (vesicle) capable of holding up to 36 S proteins. The RBCs then deliver the package of S proteins to ACE2 receptor sites in the body for recognition and binding. It seems the principal pathway used is through the spleen, where immune cells ingest (*phagocytize*) the presented immuno-pathogen.[1336]

The advantages of this approach include factors mentioned above, plus the speed with which delivery can be achieved and the lessened likelihood of side-effects. Lead author of the report, Sebastian Himbert, remarked in an interview, "We have developed a method where we can trigger an immune response without the use of genetic material and yet we are able to synthesize these particles in a very short amount of time."[1337]

[1332] Rubin, "COVID-19 Nasal Spray," discusses the development of this application to the already existing AstraZeneca vaccine. Five such sprays had reached clinical testing stages by June, 2021.
[1333] Broadfoot, "Nose Spray Vaccines Could Quash COVID Virus Variants."
[1334] For the details, see Himbert et al., "Erythro-VLPs."
[1335] For a deeper look at all the issues and potential applications, see Muzykantov, "Drug Delivery by Red Blood Cells."
[1336] Himbert et al., "Erythro-VLPs," 2–10.
[1337] Himbert quoted in McMaster University, "Researchers Re-engineer Red Blood Cells."

U. S. Investment in COVID-19 Vaccines

By any measure vaccines are a sensible investment by governments in the welfare of people, and perhaps more so than any other health care measure.

The authorization of vaccines in the United States was accompanied by significant investment. Governments not only have an inherent obligation to protect the health and wellbeing of citizens, but a vest economic interest in doing so. Sick citizens cost money and dead ones can't pay taxes or help the economy. So, for multiple reasons, government assists vaccine development.

Richard Frank (director of the USC-Brookings Schaeffer Initiative on Health Policy), with colleagues Leslie Dach (chair of Protect Our Care) and Nicole Lurie (former Assistant Secretary for Preparedness and Response (ASPR) at the United States Department of Health and Human Services (HHS)), explain in an article the government response to COVID-19: "First, it supported additional preclinical studies. Second, it absorbed the bulk of human testing costs and risk through a set of contracts that paid for the various phases of vaccine development and manufacturing. And third, it reduced manufacturing risk by underwriting capacity investments."[1338]

The following chart from the Congressional Budget Office illustrates this investment in terms of funding provided.

Box 2. Continued

Federal Funding to Support the Development of a COVID-19 Vaccine

BARDA Funding for COVID-19 Vaccines as of March 2, 2021

Sponsor	BARDA Funding (Millions of dollars)	Funding for Research and Clinical Trials?	Funding for Manufacturing?	Funding to Purchase Vaccine?	Type of Vaccine	Date Entered Phase I Clinical Trials	Date Entered Phase II Clinical Trials	Date Entered Phase III Clinical Trials	Date Received EUA	Doses to be Purchased (Millions)
Pfizer and BioNTech	5,973	No	No	Yes	mRNA	4/29/20[a]		7/27/20	12/11/20	300
Moderna and NIAID	5,896	Yes	Yes	Yes	mRNA	3/16/20	5/29/20	7/27/20	12/18/20	300
Sanofi Pasteur and GlaxoSmithKline	2,073	Yes	Yes	Yes[b]	Spike Protein	9/3/20[a]		*	*	100
Johnson & Johnson (Janssen subsidiary)	1,998	Yes	Yes	Yes	Viral Vector	7/15/20[a]		9/7/20	2/27/21	100
Novavax	1,600	No	Yes	Yes[b]	Protein Subunit	5/25/20[a]		12/22/20[c]	*	100
AstraZeneca and Oxford University	1,600	Yes	Yes	Yes[b]	Viral Vector	4/23/20[a]		8/28/20[d]	*	300
Merck and IAVI	143	Yes	Yes[e]	No	Viral Vector	8/27/20[a]		Discontinued 1/25/21		

Data source: Congressional Budget Office, using data from the Department of Health and Human Services. See www.cbo.gov/publication/57025#data.

In a bi-partisan endeavor begun first under a Republican president and sustained under a Democrat president, the federal government has been active in supporting vaccines as a principal line of defense against COVID-19. As seen in the chart above, the government provided funding to seven vaccine developers.

[1338] Frank, Dach, and Lurie, "It Was the Government that Produced COVID-19 Success."

Beginning in May, 2020, under the auspices of the Department of Health and Human Services (DHHS), 'Operation Warp Speed' pushed for the safe but rapid development and distribution of an effective vaccine against SARS-CoV-2.

Multiple governmental agencies collaborated with DHHS, including the Centers for Disease Control and Prevention (CDC), the Food and Drug Administration (FDA), the National Institutes of Health (NIH), and the Department of Defense (DOD). The funding for Operation Warp Speed was provided through the Biomedical Advanced Research and Development Authority (BARDA). Of the seven vaccine developers, six signed advance-purchase agreements. Two of the companies with vaccines that received emergency use authorizations received additional funding for selling more doses than were guaranteed by the advance-purchase agreements.[1339]

The figures in the above table, of course, need context. The average American may be staggered at the governmental investment but it does not easily translate into the cost at a smaller level. So let's consider that matter. The average cost per dose, for example, of the Moderna vaccine—one of the seven listed above—has been estimated to be $15–$16.50 in the United States and $18 per dose in Europe. That is an exceedingly modest amount for a life-saving medical intervention. This can also be put into economic context: Israel, which paid $23.50 per dose would have paid as much economically with two days of lockdown as the cost to vaccinate the entire population.[1340]

Though it is true U.S. taxpayers ultimately fund the vaccine, it is also true they do so indirectly, as they do everything else funded by tax revenue. In actual out-of-pocket expenses for the vaccine these are typically *zero* because health insurers are required to pay for them and for those without insurance the public health infrastructure provided by government provides them at no cost.

The U.S. investment in combating the pandemic has not been merely on the part of government, of course. The pharmaceutical industry—to which we can reasonably impute both profit and humanitarian motives—already had been investing heavily in research and development (R&D) *prior* to the pandemic. The U.S. Congressional Budget Office in the spring of 2021 reported the industry spent $83 billion in R&D—and noted this was, after adjustment for inflation, about 10 times what it was spending in the 1980s. On average, these companies spent about one-third of their revenue on R&D in 2019—and that represented almost twice as much of their revenue share as was true only twenty years earlier. To put this into perspective, this represents a far larger share of revenue devoted to R&D than is typically found in other 'knowledge-based' industries (e.g., those dealing with technology hardware and software).[1341]

[1339] Congressional Budget Office, "Research and Development in the Pharmaceutical Industry," 10.

[1340] Serwer and Zahn, "Guess Who Pays for All Those Millions of COVID Vaccines."

[1341] Congressional Budget Office, "Research and Development in the Pharmaceutical Industry," 1.

Despite accepting that companies are in business to make money, some Americans seem to resent pharmaceutical companies for profits made on vaccines. But as economists have pointed out, even with making a profit the *value* of the money made remains *below* the social benefit derived from their vaccines. Nobel prize-winning economist Michael Kremer has estimated the benefits from COVID-19 vaccines are somewhere between *40–300 times greater than the price being charged* by the pharmaceutical companies.[1342]

Vaccine Targets

Physician Peter Hotez, professor of pediatrics and molecular virology and microbiology at Baylor College of Medicine, relates that the research group he is part of laid the groundwork for the current vaccines with their work over the last decade. Specifically, he elaborates, "We learned that the spike protein is the soft underbelly of the virus. And we showed that if you deliver the spike protein as a vaccine, it's highly effective—induces what are called virus-neutralizing antibodies."[1343]

We saw in the chapter on the immune system that neutralizing antibodies (nAbs) are a goal of vaccines. Years before COVID-19 appeared work was going on to develop NAb vaccines against SARS-CoV and MERS. Such nAbs include:

- *monoclonal antibodies* (mAbs);
- *functional antigen-binding fragments* (fAbs); and
- *single-domain antibodies* (Nbs).

These all target the spike (S) protein, whether its receptor-binding region (RBD), or the N-terminal domain (NTD)—both subunit 1 (S1)—or the fusion and entry mechanisms of subunit 2 (S2).[1344]

The challenge of targeting the S protein is its tendency to mutate; these mutations not only characterize different variants of SARS-CoV-2 but potentially lower the effectiveness of vaccines as one or another mutation occurs. With this reality in mind there also has been investigation of targeting the M or N structural proteins because these are less prone to mutations.[1345]

A major goal in developing effective vaccines against COVID-19 is to assist the human immune system to quickly recognize SARS-CoV-2 and respond with antibodies. The intended antibodies are ones that prevent the entry of SARS-CoV-2 into cells by one or the other of two blocking actions; one targets ACE2–RBD binding interactions and the other S-mediated membrane fusion.[1346]

[1342] McDonnell and Toxvaerd, "How Does the Market for Vaccines Work?"
[1343] Berg, "How a Decade of Coronavirus Research Paved Way."
[1344] Jiang, Hiller, and Du, "Neutralizing Antibodies," 355–57.
[1345] On targeting the membrane (M) protein, see Jörrißen et al., "Antibody Response to SARS-CoV-2 Membrane Protein," 8. On targeting the nucleocapsid (N) protein, see Dutta, Mazumdar, and Gordy, "The Nucleocapsid Protein of SARS-CoV-2."
[1346] Lee et al., Antibody-Dependent Enhancement," 1185.

Unintended Consequences

One concern in creating a vaccine is the unintended consequence of antibody-dependent enhancement (ADE). With respect to SARS-CoV-2's close kin, researchers Akiko Iwasaki and Yexin Yang point out, "There is no evidence that ADE facilitates the spread of SARS-CoV in infected hosts."[1347] And ADE has not emerged as an issue in vaccination for COVID-19.

Continuing Efficacy

The three approved U.S. vaccines were developed before the more infectious and deadly Delta variant emerged. However, the original vaccines proved efficacious against Delta. Booster shots were recommended for those whose second vaccine shot had occurred six months or longer before (two months for those who received the Johnson & Johnson vaccine).

When the Omicron variant emerged late in 2021, further concern was raised about the continuing effectiveness of the vaccines. Omicron was showing itself as considerably more infectious than Delta, though perhaps less prone to produce serious illness. An early study in Scotland, where the variant was first recorded November 21, 2021, found that by December 19—less than a month later—23,840 Delta cases had been recorded (49.2% among those ages 20–39). But continuing vaccine protectiveness was demonstrated in that a booster shot was associated with a 57% reduction in risk of symptomatic infection, and hospitalization occurrences were rare (15 cases, or .06%).[1348]

Still, it is important to note that research and development of vaccines to combat COVID-19 are ongoing. For example, in late December, 2021, it was reported that the *Spike Ferritin Nanoparticle COVID-19 vaccine* (SpFN), developed at Walter Reed Hospital, had successfully completed animal trials and phase 1 human trials. This vaccine is reputed to be effective against all SARS and COVID-19 variants—including Omicron.[1349]

Vaccinated vs. Unvaccinated

As noted in an earlier chapter, research studies consistently—*persistently*—show the relatively better outcomes for those vaccinated compared to the unvaccinated.

Even against the highly contagious Omicron variant that emerged at the end of 2021—a variant that lowered vaccine effectiveness against infection—vaccines still provided significant protection against serious illness, hospitalization, and death. A study from South Africa reported in preprint in late December, 2021, examined infections and hospitalizations during the early Omicron wave. It found that of 20,021 positive cases of SARS-CoV-2, 91.4% were among the unvaccinated, and 97.1% of the hospitalizations were from members

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[1347] Iwaskai and Yang, "The Potential Danger of Suboptimal Antibody Responses," 339.

[1348] Sheikh et al., "Severity of Omicron Variant." However, the relative reduction of risk against Omicron was less than against Delta, where prevention efficacy was 83% after a booster shot.

[1349] Dutton, "Super Vaccine."

of this same group. The other 9.6% of infections were among those vaccinated (Johnson & Johnson vaccine, with booster of the same), with only 2.9% of all those hospitalized coming from the vaccinated population. Even more dramatic, those who were 1–3 months past receiving the booster accounted for only 1.5% of infections and 0.4% of hospitalizations.[1350]

Vaccine Limitations

As is true of most medical interventions, vaccines provide no guarantees. What they do well they often do spectacularly well. But they do not do all things equally well. Vaccines against COVID-19 are most successful at preventing death, then preventing serious illness, and finally in preventing infection at all. The above three things they do very well. But they do poorer at preventing transmission of SARS-CoV-2. Put bluntly, though they are excellent at the individual level in preventing infection, at the 'herd' level—across population groups—they are far less effective. Dr Tedros Adhanom Ghebreyesus, WHO Director-General, in a press conference remarked, "Vaccines save lives but they do not fully prevent transmission. Data suggests that before the arrival of the Delta variant, vaccines reduced transmission by about 60%. With Delta, that has dropped to about 40%. If you're vaccinated, you have a much lower risk of severe disease and death but you are still at risk of being infected and of infecting others."[1351]

Using data obtained from 465 facilities in a large U.S. health care database, a study released at the start of 2022 examined 1,228,664 persons who had completed a primary course of vaccination against COVID-19 (2 shots of Moderna's or Pfizer's vaccine, or 1 shot of Johnson and Johnson's vaccine), including a period of at least 2 weeks after, during which the vaccine takes full effect. Some of the results can be tabulated as follows:

Outcome	# of people	# per 10,000	Percent of Vaccinated	Percent of Infected
Infection (COVID-19)	2,246	18	0.18%	-
Hospitalized	327	2.7	0.03%	14.6%
Severe COVID-19*	189	1.5	0.015%	8.4%
Death	36	0.3	0.003%	1.6%

*Severe=hospitalization + ICU admission, acute respiratory failure, and/or noninvasive ventilation.

Every vaccinated person who died had at least one of eight critical risk factors; of those who died, 77.8% of them had at least four of these risk factors. The risk factors, with adjusted odds ratios (aORs) for severe COVID-19 indicating the greater risk compared to others, included:

[1350] Gray et al., "Vaccine Effectiveness Against Hospital Admission," Table 1.
[1351] WHO, "COVID-19 Virtual Press Conference" (Nov. 24, 2021).

- age 65 or older: aOR 3.22 (3.22x risk (322%, or more than 3-fold);
- immunosuppression: aOR 1.91 (97% greater risk);
- pulmonary disease: aOR 1.69 (69% greater risk);
- liver disease: aOR 1.68 (68% greater risk);
- chronic kidney disease: aOR 1.61 (61% greater risk);
- neurologic disease: aOR 1.54 (54% greater risk);
- diabetes: aOR 1.47 (47% greater risk); and.
- cardiac disease: aOR 1.44 (44% greater risk)/[1352]

Vaccine-Associated Enhanced Respiratory Disease (VAERD)

In the case of respiratory infections such as coronaviruses if they create ADE they can produce *enhanced respiratory disease* (ERD).

Vaccine Failure

Sometimes vaccines fail. Understanding why matters for future success. In 2012, The Council for International Organizations of Medical Sciences (CIOMS) attempted to further such an understanding by devising a classification system by which a failed vaccine was placed into one or another of four categories:

- *Confirmed clinical vaccine failure*—a fully and appropriately vaccinated person contracts the vaccine-preventable disease.
- *Suspected clinical vaccine failure*—a fully and appropriately vaccinated person apparently contracts the vaccine-preventable disease but it remains unconfirmed if it is the vaccine-specific disease.
- *Confirmed immunological vaccine failure*—a fully and appropriately vaccinated person fails to develop accepted markers of a protective immune response.
- *Suspected immunological vaccine failure*—a fully and appropriately vaccinated person apparently fails to develop accepted markers of a protective immune response, but the testing time-frame makes this uncertain.[1353]

To win approval for use a vaccine must *not* fail in terms of most people. Efficacy is a measure of this relative success; a vaccine of 50% efficacy means half the vaccinated people will contract disease when they meet the infectious pathogen. So 100% efficacy is an ideal, not a realistic goal. Influenza vaccines are recommended each year even though they typically have efficacy in the 40–60% range. But some vaccines come remarkably close to perfection. For example, both of the U.S. approved mRNA vaccines (Pfizer and Moderna) reported very high efficacy results after Phase III trials (95% and 94%, respectively).[1354]

[1352] Yek et al., "Risk Factors."
[1353] Poland, "Influenza Vaccine Failure," Table 2.
[1354] Tregoning et al., "Progress of the COVID-19 Vaccine Effort," 627 (Table 1).

How important are vaccines? It doesn't take a scientist to realize how crucial vaccines are to safeguarding public and personal health. Retired four star Army General Stanly McChrystal in an interview with MarketWatch (Nov. 16, 2021) declared his support for vaccine mandates by observing, "Go back to Gen. George Washington. He dictated the vaccine for smallpox. Had he not done that, it is unlikely that the Continental Army would have won."[1355]

The lessons of history are underscored by present real world data. In early 2022, London-based *The Financial Times* reported its analysis of the difference vaccination makes. Comparing the United States, with its 64% vaccination rate to various European nations, the paper noted how the situation in the States might be with a better vaccination rate. For example, if the U.S. had the same rate as Denmark (81%), the number of those hospitalized with COVID-19 on January 19th might have been 91,000 instead of 161,000—a difference of 70,000 (and 56% of the current number). Or, had it achieved Portugal's (90%) vaccination rate, then in the last half of 2021 the hospitalization rate might have been 39,000 a day rather than the actual 80,000.[1356]

Still, those resistant to vaccination are likely to point out that even the vaccinated become infected by highly infectious variants. But they ignore the variable outcomes healthwise between those unvaccinated an those vaccinated. In an interview on the *Today* show, noted physician Vin Gupta said, "We have to get comfortable with fully vaccinated folks testing positive, that's going to be our new normal. But people should not worry about that because *the purpose of vaccines is not to prevent a positive test or a respiratory virus like omicron, it's to keep you out of the hospital*, and that's exactly what they're doing."[1357] Vaccines don't guarantee immunity from infection, but they greatly improve the odds that if infection occurs the result will be more favorable to the infected.

Professor of Emergency Medicine Phillip Coule put the stark contrast between the vaccinated and unvaccinated this way in an interview: "The truth is that an 80-year-old that's vaccinated and boosted and gets Covid most of the time has nothing more than a cold; a healthy 50-year-old who's a little bit overweight, has problems with blood pressure or diabetes and is not vaccinated at all ends up in the ICU."[1358]

[1355] McChrystal quoted in Price, "'Society's Very Existence Could Have Been Threatened.'"
[1356] Barnes, Burn-Murdoch, and Smythe, "'Pandemic of the Unboosted.'"
[1357] Gupta's remarks on the *Today* show (Dec. 16, 2021) reported by Mazziotta, "Doctor Says." Italics added for emphasis.
[1358] Coule quoted in Barnes, Burn-Murdoch, and Smythe, "'Pandemic of the Unboosted.'"

Chapter 8
Three Vaccines

mRNA Vaccines (Moderna & Pfizer)

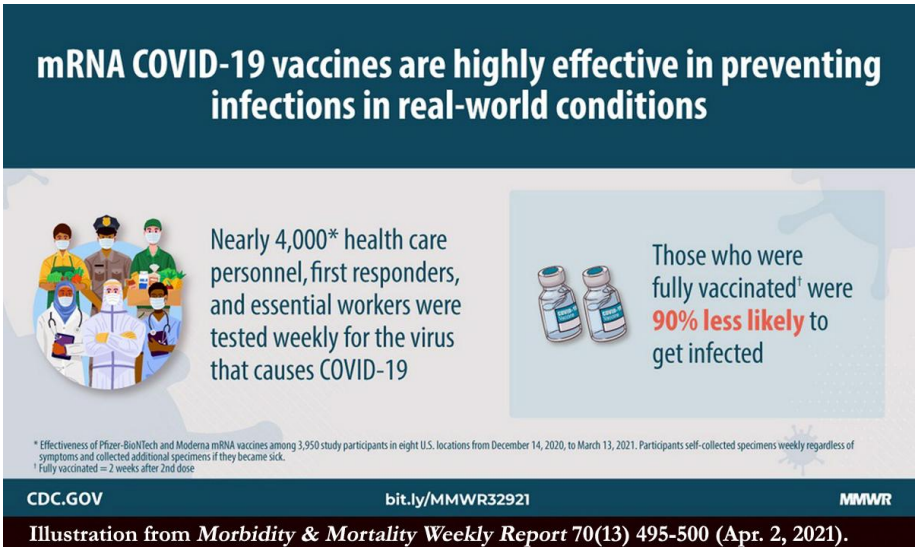

Illustration from *Morbidity & Mortality Weekly Report* 70(13) 495-500 (Apr. 2, 2021).

The mRNA vaccines are widely regarded as one of the most remarkable and successful achievements of medical science in a long time. Taking advantage of technical advances in the last few decades, and the store of knowledge already acquired about coronaviruses (see chapter 2), the developers of mRNA vaccines were able to respond very rapidly to the crisis posed by the COVID-19 pandemic. Still, many Americans were skeptical about how much was known.

A Brief History Lesson

In fact, we may forgive those who wonder if Americans are ever satisfied when it comes to vaccines. For example, one of the concerns raised at the start of the pandemic was how quickly a vaccine could be readied. But when mRNA vaccines were readied in less than a year, a different concern was voiced—how could they be safe having been so rapidly developed? It must seem to outsiders that Americans cannot be pleased no matter what science establishes.

Of course, for those concerned about the speed with which vaccines appeared, there seemed to be grounds for suspicion. After all, development of vaccines historically has taken place at a plodding pace—sometimes decades—and the previous record for development was often cited as *four years*. Antivaxxers were quick to try turning concern over rapid development of vaccines into fear, beating the drum about evil Big Pharma's profiteering motive and lack of concern for people's health while offering as their counter measure their own commercial products, many of them unproven and some actually known to be

unsafe. But doubt-turned-into-fear motivates and by early 2021, some two-thirds (67%) of those who said they definitely or probably wouldn't get the vaccine named concern over its rapid development as a reason why.[1359]

Fear can be challenged by facts. First, the antecedent research for COVID-19 vaccines is almost a century old. Second, in addition to some 90 years of previous study of coronaviruses, the technological development of being able to rapidly decode a virus' genome has been a critical factor in the speed by which vaccines can be developed.[1360] Third, another technological advance of great importance has been the ability to rapidly share data among scientists—something far harder just a half-century ago.[1361] As a side note, it might be mentioned that while it is frequently said that the previous record for a vaccine was four years, the urgency of the Ebola virus crisis in 2014 spurred vaccine development such that in less than a year a dozen clinical trials had moved from a 'first in man' dosing study to phase III efficacy trials.[1362] The keys then were much the same as in 2020: advanced technology and widespread collaboration.

It may be helpful to examine a timeline for mRNA vaccine development.

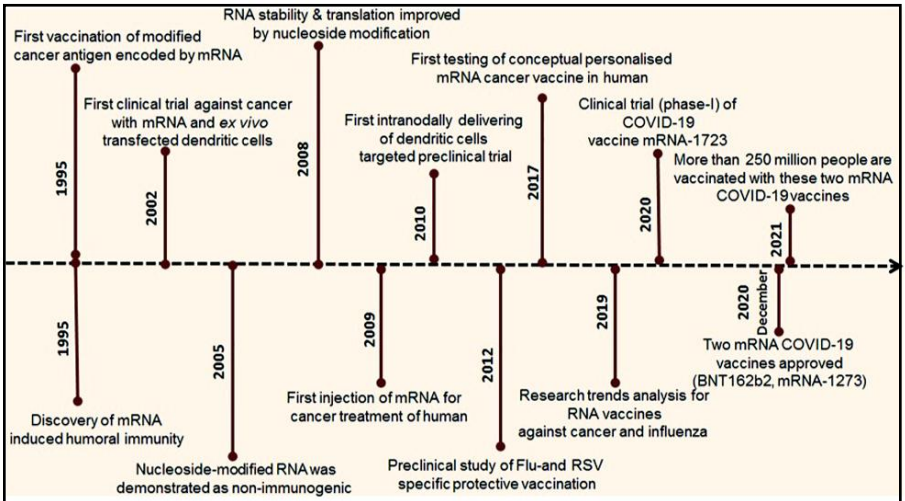

FIGURE 2 | Timeline of the research breakthrough and progression of mRNA vaccine.

Illustration from Chiranjib Chakraborty, Ashish Ranjan Sharma, Manojit Bhattacharya, and Sang-Soo Lee. "From COVID-19 to Cancer mRNA Vaccines: Moving from Bench to Clinic in the Vaccine Landscape." *Frontiers in Immunology* 12 (July, 2021) art. 679344. doi: 10.3389/fimmu.2021.679344. The material is from p. 3 and is made available by Creative Commons Attribution (CC BY) License (https:// creativecommons.org/licenses /by/4.0/ legalcode). Changes have been made to font type in the illustration's explanatory text.

[1359] Funk and Gramlich, "10 Facts about Americans and Coronavirus Vaccines."
[1360] Ellis, et al., "Decoding Covid-19."
[1361] Le Guillo, "Covid-19."
[1362] Petousis-Harris, "Assessing the Safety of COVID-19 Vaccines," 1205. Also see Branswell, "'Against All Odds.'"

But while the timeline might give the impression that things only got rolling in the mid-1990s, the truth is that relevant research is older than that. For example, *messenger RNA* (mRNA) was discovered in 1960, and *liposomes* (the spherical vesicles commonly used to package drugs) were first described in the mid-1960s. These two components would prove key to developing a mRNA vaccine. Liposomes were first employed in vaccine delivery in 1974, and just four years later were used to deliver mRNA to cells (murine and human). An important breakthrough occurred when the delivery of mRNA wrapped in *cationic liposomes* (synthetic carriers of nucleic acids like RNA) was shown to produce proteins in human cells in 1989.[1363]

Other important work on developing mRNA technology—beside that shown in the timeline—proceeded in the last decade of the 20th century and first two of the 21st century. In an article published in 2018, microbiologist Norbert Pardi and colleagues pointed out that "for most emerging virus vaccines, the main obstacle is not the effectiveness of conventional approaches but the need for more rapid development and large-scale deployment." They observed that the first published report of successful use of nucleic acid therapeutics was in 1990. They also pointed out that in the period between 2008–2018 both major investment and technological innovation had enabled mRNA technology to emerge as a promising tool in vaccine development.[1364]

For instance, in 2000, the first four-component *lipid nanoparticle* (LNP) was successfully developed. LNPs have been described as 'tiny balls of fat'[1365] and they are instrumental in protecting fragile nucleic acid material for transport into cells. Then, in 2005, it was demonstrated that modified mRNA could escape immune system detection. In 2012, a murine mRNA vaccine appeared. Finally, in 2015, the first LNP-mRNA vaccine for humans was developed.[1366] All these various developments are why researchers, in the months prior to the identification of SARS-CoV-2 and the start of the pandemic, noted that in the previous two decades mRNA vaccines had been investigated extensively both for use against infectious diseases and cancer, and indicated that such vaccines were being developed with both prevention (*prophylactic* use) and treatment (*therapeutic* use) in mind.[1367]

Earlier, it was pointed out that mRNA vaccine developers were able to respond to COVID-19 rapidly. For example, Moderna's product vaccinated the first trial participants on March 16, 2020—just 66 days after the SARS-CoV-2 genome was made public (January 10, 2020). Amazingly, drawing on existing genomic data, the complete mRNA code for the vaccine was compiled in 2 days; within 45 days the materials required for clinical trials had been manufac-

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[1363] Jawalagatti, Kirthika, and Lee, "Oral mRNA Vaccines," 2 (esp. Fig. 1).
[1364] Pardi et al., "mRNA Vaccines," 261.
[1365] Cross, "Without These Lipid Shells."
[1366] Jawalagatti, Kirthika, and Lee, "Oral mRNA Vaccines," 2 (esp. Fig. 1).
[1367] Zhang et al., "Advances in mRNA Vaccines," 4.

tured and delivered.[1368] The rest, as they say, is history: by the end of the first week of June, 2022, there had been 222,237,499 doses of the Moderna vaccine administered in the United States.[1369]

Advantages of mRNA Vaccines

Let's start with the bottom line: mRNA is, genetically, a 'minimal construct' and as such it presents only those elements required for expression of the encoded protein—and it does not interact with the genome, so it presents a very safe profile.[1370] It is a common refrain in the literature to point out the advantages of mRNA vaccines. Here are some suggested in the scientific literature.[1371]

Compared to . . .	mRNA vaccines . . .
whole bacteria, subunit, and DNA vaccines	can induce T-cell and B-cell immune response, are non-integrating and naturally degradable, and pose a lower risk of virulence reversion, and insertional mutagenesis.
DNA, subunit, killed and live attenuated virus vaccines	are a non-infectious, non-integrating platform, posing no potential risk of infection or insertional mutagenesis.
DNA and viral vectored vaccines	are non-infectious, non-integrating, natural degradation, egg and cell free, rapid and scalable production; stimulation of innate immune response; induction of T and B cell immune response.

The repetition in the Table is to make the point as obvious as possible: compared to other kinds, mRNA vaccines offer many significant advantages.

However, mRNA vaccines pose challenges. For example, early in the distribution of such vaccines the need to store them at very cold temperatures exceeding the normal capabilities of many facilities was an issue that had to be resolved quickly to make administration more feasible. Any number of issues unforeseen before real-world application have had to be identified and addressed, even as research continues to further development of the vaccine itself.

[1368] Granados-Riveron and Aquino-Jarquin, "Engineering of the Current Nucleoside-Modified mRNA-LNP Vaccines," 1.

[1369] CDC, "COVID Data Tracker" (June 6, 2022; https://covid.cdc.gov/covid-data-tracker/#vaccinations_vacc-total-admin-rate-total.)

[1370] Schlake et al., "Developing mRNA-Vaccine Technologies," 1319.

[1371] Table rows are from, respectively, Jin et al., "mRNA Vaccine," 2, 4; Pardi et al., "mRNA Vaccines," 261; and Zhang et al., "Advances in mRNA Vaccines," 3.

Making an mRNA Vaccine: An Overview

The development of *messenger RNA* (mRNA) *vaccines* has been a remarkable advancement in vaccine technology. The logic of such vaccines is simple: instruct body cells through a short-lived message (the mRNA) to make a certain protein that the immune system will recognize as a potential danger and mount a defense against it. This mRNA message can be produced in the laboratory and rapidly reproduced—much more rapidly than is true for vaccines prepared in older, traditional ways.

However, what is new and unknown often provokes anxiety, and many people have expressed anxiety about the ingredients in the new vaccines. The ingredients can be divided into two kinds:

- *active*—the mRNA 'instruction kit'; and,
- *inactive*—those ingredients to transport and support the mRNA.

The active ingredient is the most important part because it is what facilitates immunity.

Let's begin with a snapshot illustration showing the basic nature of both approved mRNA vaccines—and one important difference.

Table 1. Current human trials for SARS-CoV-2 using mRNA lipid nanoparticles. All mRNA vaccines in SARS-CoV-2 clinical trials use a lipid nanoparticle for delivery.

Company	mRNA Type	Immunogen	mRNA Dose (μg)
Moderna	nucleoside modified mRNA	membrane bound prefusion stabilized spike	100
BioNTech Pfizer	nucleoside modified mRNA	membrane bound prefusion stabilized spike	30

The illustration is extracted from one in Michael D. Buschmann, Manuel J. Carrasco, Suman Alishetty, Mikell Paige, Mohamad Gabriel Alameh, and Drew Weissman. "Nanomaterial Delivery Systems for mRNA Vaccines." *Vaccines* 9 (2021) 65. https://doi.org/10.3390/vaccines9010065. The material appears on page 3 and is made available by Creative Commons Attribution (CC BY) License (https:// creativecommons.org/licenses /by/4.0/ legalcode). Changes have been made to font type in the illustration's explanatory text, which has been altered to exclude all text beyond the first two sentences. There are two columns to the right of those shown which have not been included and five rows of other companies and their vaccines that have been excluded here. *Also see* Granados-Riveron and Aquino-Jarquin, "Engineering of the Current Nucleoside-Modified mRNA-LNP Vaccines against SARS-CoV-2," 5 (Table 2).

Some notes on the above Table material will help prepare us for a more in-depth look at mRNA vaccines in general, and then the two approved ones in particular. First, the mRNA type is the same—'nucleoside-modified mRNA.' A *nucleoside* is a biological molecule that can be a precursor to nucleotides (a nucleoside plus a phosphate group) needed for RNA synthesis. *Nucleoside-modified mRNA* is messenger RNA (mRNA) that has been changed by human technology. Native nucleosides (e.g., *uridine*) might be replaced by chemically modified versions (e.g., *pseudouridine*) in order to enhance efficiency in the mRNA being

translated inside the cell *and* to either enhance or reduce its immunogenicity (ability to provoke an immune response).

Next, the phrase 'nucleoside-modified mRNA' can profitably be preceded by the abbreviation 'LNP,' for both vaccines are *LNP-encapsulated*. This means they are enclosed ('encapsulated') within a *lipid nanoparticle* (LNP)—a very, very small ('nanoparticle') package made of lipids ('fats'). The nature of these LNPs and their purpose are discussed below in the section on the inactive ingredients of the vaccines.

Third, the *immunogen* is the specific *antigen*, or immune system prodding agent. It is the 'active' ingredient in that its action is what prods the human system to notice and do something about what it is being shown. In both vaccines this immunogen is the same: 'membrane-bound prefusion stabilized spike.' As we are familiar with now, *spike* refers to the spike (S) protein on the surface of SARS-CoV-2 that it uses to attach to a host cell and which facilitates its entry into the host. Keeping this S protein in a *prefusion stabilized* state prevents it from undergoing the changes that facilitate viral fusion with the cell membrane. This also proves to be a potent provoker of immune response (the 'immunogen').[1372] We shall examine this further in the section on the vaccine's active ingredient.

Fourth, the *dose* used by each mRNA vaccine is different. Moderna provides 100 μg. of nucleoside-modified mRNA in a 0.5 mL dose, while Pfizer's is 30 μg. of the active ingredient. Although the desire is always to use the least dose that achieves the desired effect what that dose is for SARS-CoV-2 and COVID-19 remains unresolved. However, by late Summer, 2021, a number of studies were finding differences in performance between the two vaccines in favor of Moderna's and it seemed plausible those differences might be accounted for by dosage size.[1373]

Finally, we might add that both mRNA vaccines share a similar though not identical schedule. Both utilize two shots—intramuscular injections—either spaced 21 days apart (Pfizer), or 28 days apart (Moderna).[1374] The spacing of doses is related to *immunogenicity*, which we have described above as the ability to provoke an immune response. It is a measure of how well a vaccine works and includes both the kind of responses generated and their magnitude.[1375] A group of medical researchers reported in the *Journal of the American Medical Association* (JAMA) in early 2022 that longer mRNA vaccine dosing intervals demonstrated improved immunogenicity. In fact, modeling studies suggested decreased mortality rates with a delayed second dose (though this might not be advisable for those in higher at-risk groups).[1376]

[1372] For an overview, see Hsieh et al., "Structure-Based Design of Prefusion-Stabilized SARS-CoV-2 Spikes."

[1373] Manjarrez, "Moderna vs. Pfizer: Is There a 'Best' mRNA Vaccine?"

[1374] Granados-Riveron and Aquino-Jarquin, ""Engineering of the Current Nucleoside-Modified mRNA-LNP Vaccines," 5 (Table 2).

[1375] AstraZeneca, "What Does Immunogenicity Mean in the Context of COVID-19 Vaccines?"

[1376] Grunau et al., "Immunogenecity of Extended mRNA SARS-CoV-2 Vaccine Dosing," 280.

Basic Steps in Making Modified-Nucleoside mRNA

Steps*	Comment
1. Gene for spike (S) protein of SARS-CoV-2 is modified as desired to create encoded antigen.	Sometimes only a portion of the S protein, such as the receptor-binding domain (RBD) is used.
2. Inscription of complementary DNA (cDNA) to create a plasmid DNA (pDNA) template.	cDNA is a DNA copy of mRNA produced by reverse transcriptase. Plasmid DNA occurs naturally (e.g., in bacteria).
3. The modified S protein antigen-gene is spliced into the plasmid.	The gene for the S protein is foreign DNA spliced into the plasmid.
4. The circular plasmid molecule (pDNA) template is linearized to serve as a template for the DNA-dependent RNA polymerase of the next step.	*Linearization* is a cutting that produces a linear molecule with single-strand 'sticky' ends.
5. The linearized pDNA is transcribed into mRNA. The transcription reaction includes the *template* (linearized plasmid DNA encoding the mRNA), a recombinant RNA polymerase, and nucleoside triphosphates.	*Transcription* occurs using bacteriophage RNA polymerase (T7 bacteriophage, a virus which infects *E coli* bacteria). Transcription encodes the instructions for making the desired protein.
6. The mRNA transcript is purified.	The plasmid and bacterial DNA has been digested by a deoxyribonuclease (DNase) enzyme. Contaminants alongside the mRNA are removed through precipitation and extraction steps. Any additional contaminating RNA material (e.g., double-stranded RNA (dsRNA) is also removed. [1377]

*The order is a bit arbitrary as pDNA templates are likely already at hand when a gene is selected to be modified and inserted. The order here is to highlight the focus on the S protein.

These can be grouped as involving 'upstream' processing (steps 1–5) for enzymatic generation of mRNA and 'downstream' processing (step 6) to purify it.[1378]

[1377] The Table relies on multiple sources, including especially, Liang, Huang, and Liu, "Development and Delivery Systems of mRNA Vaccines," 2–3; Rosa et al., "mRNA Vaccines Manufacturing," 2192–94; Schlake et al., "Developing mRNA-Vaccine Technologies," 1319–20; Zhang et al., "Advances in mRNA Vaccines," 2.

[1378] Rosa et al., "mRNA Vaccines Manufacturing," 2194.

Both of the approved mRNA vaccines are 'conventional' ones using mRNA techniques developed before the pandemic. In both the goal is an mRNA encoded full-length spike (S) protein, which consists of the SARS-CoV-2 glycoprotein with a transmembrane anchor and an intact S1–S2 cleavage site, but with the spike configuration fixed in a prefusion state to stabilize it in an optimal antigenic presentation.[1379] The S protein structure looks like this:

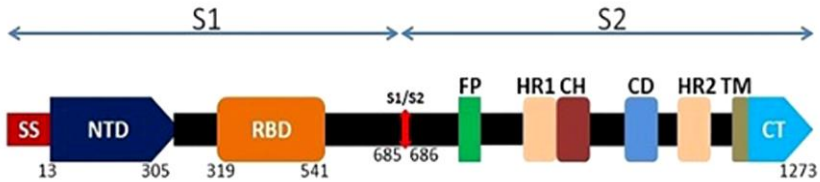


FIGURE 1 | Schematic genomic organization of the full length spike glycoprotein of SARS-CoV-2. The surface spike protein contains S1 and S2 domain. At the N terminal, S1 domain consists of a signal sequence (SS) which is followed by N-terminal domain (NTD; 293 amino acids) and Receptor Binding Domain (RBD; 222 amino acids). S2 domain consists of fusion peptide (FP) followed by two heptad repeats (HR1 and HR2), a central helix (CH), a connector domain (CD), a transmembrane domain (TM) and the cytoplasmic tail (CT).

Illustration is from Yashpal S. Malik, Prashant Kumar, Mohd Ikram Ansari1 Maged G. Hemid, et al. "SARS-CoV2 Spike Protein Extrapolation for COVID Diagnosis and Vaccine Development." *Frontiers in Molecular Biosciences* 8 (July, 2021) art. 607886. doi: 10.3389/fmlob.2021.607886. Material appears on page 7 and is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/legalcode). The original has been changed in font size and type for explanatory material.

This step is the 'first' one in the sense of representing the specific aim of a SARS-CoV-2-specific mRNA vaccine. However, it depends on plasmid DNA (pDNA) and the processes associated with plasmid. Researchers Yongjun Liang, Liping Huang, and Tiancai Liu explain:

> mRNA is usually synthesized using linearized plasmid DNA containing the ORF encoding the target protein as a template and is generated by *in vitro* transcription, with the 5' cap and poly(A) tail added at the end of the process. By including the 5' and 3' UTRs, poly(A) tail, 5' cap and nucleoside triphosphate (NTP) on the DNA template for in vitro transcription of the mRNA vaccine, the mRNA synthesis elements such as the 5' cap and NTP can be specifically designed to improve the stability and translation efficiency of the mRNA.[1380]

We will return to the subject of the S protein shortly.

[1379] See Ura et al., "New Vaccine Production Platforms," 198.
[1380] Liang, Huang, and Liu, "Development and Delivery Systems of mRNA Vaccines," 2–3.

In describing the role of plasmid here we will also stretch back and forth across the whole process. While there is a sequence entailed it is easy to get caught up in a particular feature and lose sight of the flow that is the whole process—or put in more familiar terms, we can lose sight of the forest by staring too hard at separate trees. For all the possible variations that can result in a mRNA vaccine, the process as a whole entails basic, identifiable steps.

Plasmid DNA (pDNA) is a small, circular section of DNA found in some microorganisms such as bacteria and commonly called, simply, *plasmid*. It is not the same as the more famous chromosomal DNA (cDNA); pDNA typically contains only a few genes and is both physically separate from cDNA and replicates separately. Plasmids have two especially valuable characteristics that vaccine manufacturers draw upon. First, they very reliably contain the target gene sequence (for the spike (S) protein—see at right). Second, they are easy to replicate and when they do so they copy the spliced-in gene. These features contribute to the rapid development and testing of mRNA vaccines.

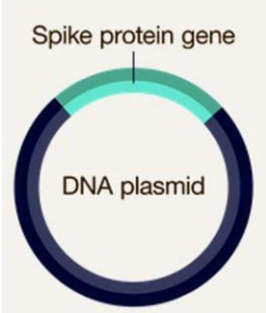


Image courtesy of National Human Genome Research Institute (https://www.genome.gov)

Making an mRNA vaccine requires significant quantities of DNA as a starting template for an enzymatic reaction for mRNA production. Near the beginning of the pandemic as manufacturers were scrambling to prepare vaccine for delivery, it was estimated that delivering one billion doses of an mRNA vaccine would require production of in excess of 1 kilogram (kg) of DNA—roughly the equivalent of 1 litre of water. At the time, the world's leading manufacturer of plasmid DNA observed that over half of the then existing pDNA manufacturing capacity would be required to meet the needs of a single mRNA vaccine manufacturer's production.[1381]

As subsequent events proved, the obstacle was not insurmountable. Moreover, because new plasmids can be produced in short order (within two weeks) a vaccine manufacturer can either test new ones looking for a best fit, or having found such a best fit reproduce quantities quickly, which leads to rapid distribution.

After the modified S protein antigen-gene is spliced into the plasmids (step 3 of the Table), these are placed into the cells of *E. coli* bacterium. The multiplying bacteria are cultured overnight in *Luria Broth* (LB) *medium* (e.g., 100 milliliters (mL)) at 37° Celsius. Then they are moved to a culture solution where they will be fermented over a span of days. Following this, the *E. coli* bacteria must be broken apart to release the plasmids.[1382]

[1381] Ohlson, "Plasmid Manufacture Is the Bottleneck," 1892.

[1382] Liang, Huang, and Liu, "Development and Delivery Systems of mRNA Vaccines," 2.

These newly harvested plasmids must undergo a quality check to ensure the intended gene sequence remains intact. Those passing this test are linearized by enzymatic action (step 4). An *alkaline phosphate* (ALP or AKP) is used for this purpose. Linearization separates the antigen-gene, which must be purified and tested again.[1383]

Then comes step 5, described a little bit below in greater detail.

Vaccine manufacturers use long-established laboratory procedures to create mRNA. In fact, they mimic nature to produce the mRNA in a similar fashion.

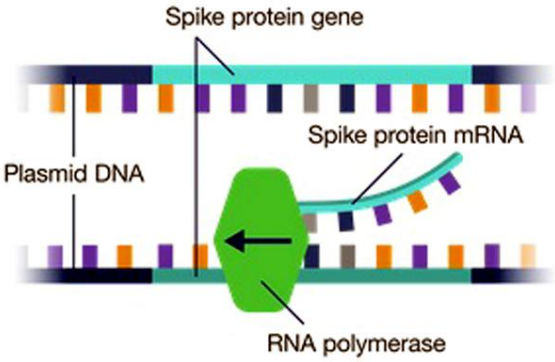


Image courtesy of National Human Genome Research Institute (https://www.genome.gov)

Simply put, as outlined in the Table for steps 2–5, they use a plasmid (pDNA) template into which they splice the gene for the spike (S) protein. This template must be produced in advance, usually by linearization of a purified plasmid (as described above), or by amplification of the region of interest using *polymerase chain reaction* (PCR), a technique that rapidly 'amplifies' via making many copies.[1384] After separating the strands of the plasmid DNA, the RNA polymerase agent that copies (*transcribes*) RNA from the DNA (as we saw in chapter 1) employs the artificial S protein to create a single mRNA molecule. Since this is all that the vaccine makers want, the remainder of the plasmid is broken down (step 6 in the Table). All that remains for the vaccine is the desired mRNA.

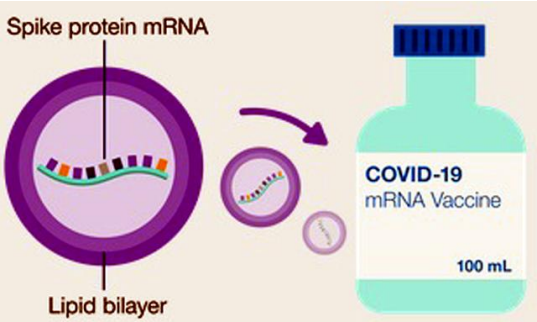


Image courtesy of National Human Genome Research Institute (https://www.genome.gov)

The manufactured mRNA is not just very small but also incredibly fragile. Both to protect and preserve it, vaccine manufacturers place it inside a *fatty lipid bilayer* delivery system known as a *lipid nanoparticle*. Not only does this covering membrane protect the mRNA, but it has the added advantage of being readily appropriated by human cells once administered via the vaccine shot.

[1383] Liang, Huang, and Liu, "Development and Delivery Systems of mRNA Vaccines," 2.
[1384] Rosa et al., "mRNA Vaccines Manufacturing," 2194.

The linearized pDNA template now takes a backseat to an important process—*in vitro transcription* (IVT) enzymatic reaction used to generate mRNA. IVT requires a number of ingredients:

- an RNA polymerase;
- nucleotide triphosphates (NTPs) substrates;
- the polymerase cofactor MgCl2;
- a pH buffer containing polyamine; and.
- antioxidants.[1385]

IVT can utilize any one of a number of polymerases (e.g., T7, SP6 or T3 RNA) to catalyze the synthesis of the target mRNA from the corresponding DNA template.[1386] Commonly, the transcribing of linearized pDNA into mRNA utilizes the *T7 RNA polymerase*, whose genome was first sequenced and annotated in the 1980s. This enzyme transcribes DNA genes into mRNA. When combined with a T7 *promoter*—a sequence of DNA 18 base pairs long that acts as a 'start' signal—the result is production of sizable quantities of RNA from a desired gene.[1387] Both the Moderna and Pfizer vaccines use T7.[1388]

But IVT entails more than just a polymerase. It also needs *nucleotide triphosphates* (NTPs) as substrates for the polymerase, a *polymerase cofactor* (magnesium chloride ($MgCl_2$)) to enhance the enzymatic activity of the polymerase (and thereby boost amplification), and a *pH buffer* for a suitable chemical environment. This buffer contains polyamine to reduce the influence of impurities. The mix is rounded out with antioxidants.

During IVT, nucleoside-modification takes place. Although it will be described further a bit later, here we will note that a primary purpose is to stabilize the resulting mRNA. Key substitutions of naturally occurring nucleosides with synthetic ones also suppress the host's natural inclination to rapidly degrade the mRNA, allowing it to persist intact to reach ribosomes in the cytoplasm.

One of the attractions of mRNA vaccine technology is its efficient use of time. In just a few hours an IVT reaction can generate milligram quantities of mRNA.[1389] Given the incredibly tiny nature of mRNA a *milligram*—though itself a very small amount—represents a lot of mRNA.

If it has not happened during IVT, the transcribed mRNA next experiences a second enzymatic reaction to produce the desired 5'-cap. This reaction is catalyzed by the *vaccinia capping enzyme*, which facilitates attachment of the 5'-cap to the mRNA molecule.[1390] All elements add together in the following illustration.

[1385] Rosa et al., "mRNA Vaccines Manufacturing," 2194.
[1386] Rosa et al., "mRNA Vaccines Manufacturing," 2194.
[1387] Walsh, "The Science Behind the Shot."
[1388] Park et al., "mRNA Vaccines for COVID-19: What, Why, and How," 1450 (Table 2).
[1389] Jin et al., "Perspectives and Prospects on mRNA Vaccine Development," 3993.
[1390] Jin et al., "Perspectives and Prospects on mRNA Vaccine Development," 3993.

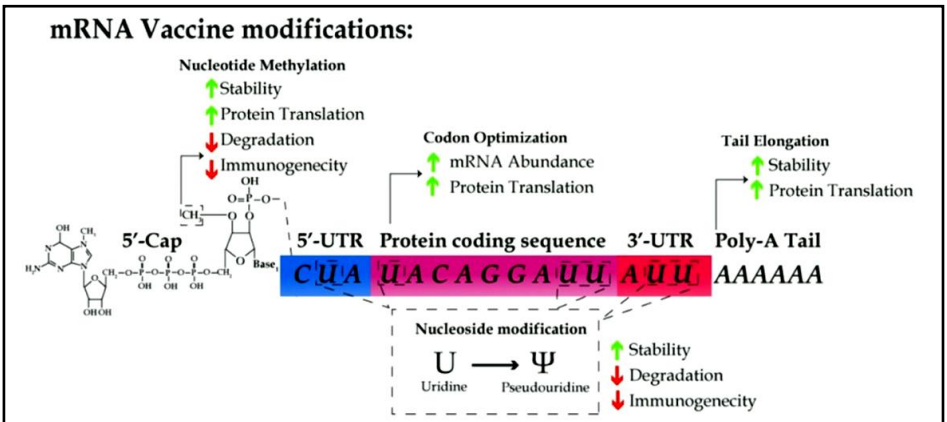


Figure 1. The schematic for designing an mRNA vaccine. mRNA molecules are synthesized in vitro with cap1 structure (m7GpppNm), the substitution of uridine with pseudouridine, and the use of the preferred codon in humans, optimized UTR and polyA tail sequence. These modifications results in the increase of RNA stability and translation efficiency as well as the reduction of immunogenicity.

Illustration is from Jung Woo Park, Philip N.P. Lagniton, Yu Liu, and Ren-He Xu. "mRNA Vaccines for COVID-19: What, Why, and How." *International Journal of Biological Sciences* 17/6 (2021) 1446–60. doi: 10.7150/ijbs.59233. Material appears on page 1450 and is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/legalcode). The original has been changed in font size and type for explanatory material.

Step 6: Purification

At the end of step 5 what is left is a mix containing more than just the desired mRNA. The impurities—e.g., enzymes, residual unnatural nucleosides (NTPs) and the pDNA template, as well as aberrant mRNAs formed during the IVT)—must be removed and not one way suffices for all. One technique used is *size exclusion chromatography* (SEC), which uses gravity flow to separate RNA molecules by size. But it is unable to remove double-sided RNA (dsRNA).[1391] Removing dsRNA can increase mRNA's translation by ribosomes significantly. More than one process exists to accomplish this, for example, *fast protein liquid chromatography* (FPLC) or *high-performance liquid chromatography* (HPLC),[1392] or *ion pair reverse-phase chromatography* (IPC). [1393]

Concluding Caution

Please note that the above is greatly simplified. The process, for example, that Pfizer uses entails some 19 steps.[1394] Exact details of important things are intellectual property that pharmaceutical companies are understandably reluctant to share. Our depiction here is a very general one.

[1391] Rosa et al., "mRNA Vaccines Manufacturing," 2195–96..
[1392] Zhang et al., "Advances in mRNA Vaccines," 2–3.
[1393] Rosa et al., "mRNA Vaccines Manufacturing," 2196..
[1394] Liang, Huang, and Liu, "Development and Delivery Systems of mRNA Vaccines," 2.

The Active Ingredient: mRNA

The *active* ingredient is the key agent meant for human immune response. The formal name for this is 'Nucleoside-modified mRNA encoding the viral spike (S) glycoprotein of SARS-CoV-2.' This active ingredient is of the same type in both mRNA vaccines. This key ingredient has two important advantages over many older vaccines: first, it does not use live or whole viral particles, and second, it non-infectious.

Let's start at the end of this phrase—'the viral spike (S) glycoprotein of SARS-CoV-2.' The first point made in the previous paragraph bears repeating and emphasis: the mRNA spike (S) protein gene is artificially manufactured; *no live SARS-CoV-2 is used*. This makes it different from some past vaccines. Of course, this is a reality anti-vaxxers would like to use against it—just as they would if the vaccine did use 'natural' live protein. A common set of myths spread about this active ingredient is that it has been manufactured to advance some dark, nefarious scheme, such as controlling people's genetic code. But mRNA, as we've seen, is *not* the same as DNA and can't combine with DNA to change the human genetic code.[1395]

In looking at the steps in building a conventional mRNA vaccine we started with an illustration of the genome structure of the S protein. Let's look again, though in a somewhat different way. Naturally occurring mRNA looks like this:

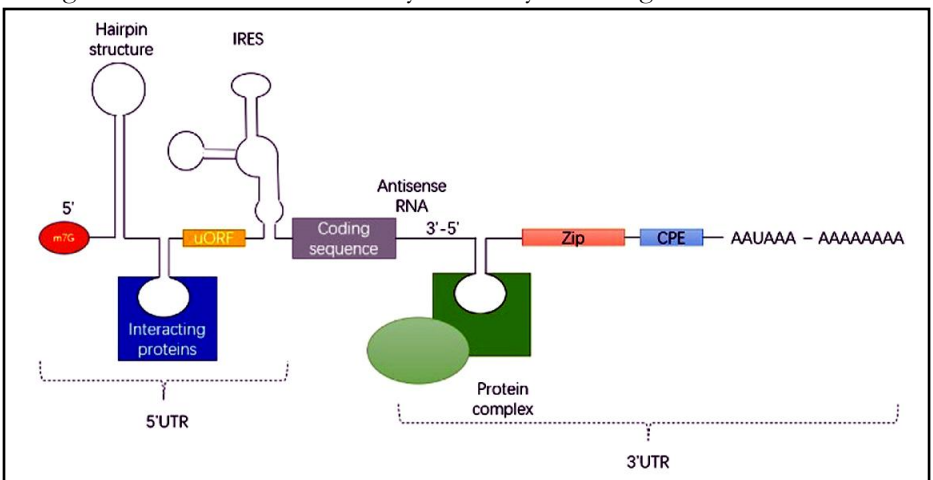


FIGURE 2 | Universal structure of eukaryotic mRNA, showing the structure of the 5' and 3' UTRs.

The illustration is from Yongjun Lian, Liping Huang, and Tianchai Liu. "Development and Delivery Systems of mRNA Vaccines." *Frontiers in Bioengineering and Biotechnology* 9 (July, 2021) art. 718753. doi.org/10.3389/fbioe.2021.718753. The material appears on page 4 and is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/ legalcode). Changes have been made to font type in the illustration's explanatory text.

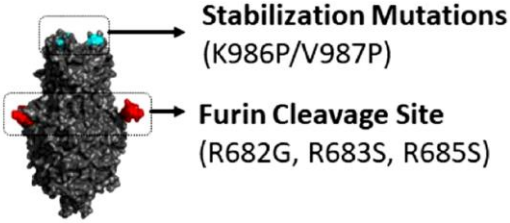
[1395] See Bolich, *Understanding COVID-19 Vaccination Arguments*, 70–73; Geddes, "Will an mRNA Vaccine Alter My DNA?"

While this ventures us deeper into the pool, the above illustration offers a closer look at what vaccine manufacturers aim at. Although manufactured in a laboratory, the mRNA is engineered to resemble fully processed, mature, *natural* mRNA molecules.[1396] The modified mRNA—synthetic mRNA (i.e., mRNA created in the lab through technology)—has three basic ingredients:[1397]

'Cap'	Open Reading Frame (ORF)	'Tail'
7-methyl-guanosine residue joined to the 5'-end via a 5'-5' triphosphate	This encodes the desired protein.	poly(A) tail at the 3'-end

A conventional mRNA vaccine includes an ORF (for the antigen desired), flanked by untranslated regions (UTRs) and with a terminal poly(A) tail.[1398] Although attention rightly focuses on the encoding of the desired protein (the *encoded protein*), the sequences of cap and tail matter, too, as well as the modified nucleosides, the LNP, the purification process, and so forth.[1399]

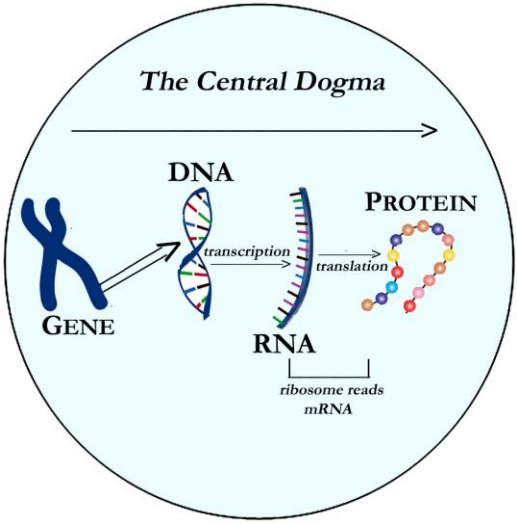
The mRNA vaccines produce a full-length spike (S) protein of SARS-CoV-2. The S protein made, though, is designed with mutations (K986P and V987P) to ensure it remains in a prefusion posture, which is favorable for presenting the antigenic appearance the immune system will respond to[1400] (at right).



Excerpted from Timothy P. Riley, Hui-Ting Chou, Ruozhen Hu, Krzysztof P. Bzymek, et al. "Enhancing the Prefusion Conformational Stability of SARS-CoV-2 Spike Protein through Structure-Guided Design." *Frontiers in Immunology* 12 (Apr., 2021) art. 660198. doi: 10.3389/fimmu.2021.660198. This image is excerpted from section A of Figure 2 on page 6 and is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/legalcode).

Naked mRNA is quickly degraded by extracellular *ribonucleases* (RNases), enzymes that break RNA molecules apart. This mRNA instability must be addressed because it has to stay intact to reach target cell cytoplasm where it can be translated by ribosomes. Thus vaccine makers must introduce one or more chemical groups to the *in vitro-transcribed* (IVT) mRNA. This process yields *modified mRNA* (mod-RNA), with the modifications specifically producing nucleoside-modified mRNA.[1401]

[1396] Pardi et al., "mRNA Vaccines—A New Era in Vaccinology," 262.
[1397] Schlake et al., "Developing mRNA-Vaccine Technologies," 1320.
[1398] Zhang et al., "Advances in mRNA Vaccines," 2.
[1399] Sanders, "Low-Dose, Single-Shot COVID-19 mRNA Vaccines Lie Ahead," 1945.
[1400] Nance and Meier, "Modifications in an Emergency," 748.
[1401] Jin et al., "Perspectives and Prospects on mRNA Vaccine Development," 3992–93.



Earlier in this chapter we saw that nucleoside-modified mRNA is messenger RNA (mRNA) that has been changed by human technology. There are dozens of ways that RNA can be chemically modified; the collection of these is known by the label 'epitranscriptome.' But before we examine more closely how this applies to mRNA vaccines, let's recall the basic process in cells: a gene—i.e., a segment of DNA code—undergoes transcription to produce a complementary RNA strand, which as 'messenger' RNA a ribosome in the cell can translate to make a protein.

Just as DNA and the proteins in the nucleus that associate with it (*histone proteins*) can experience reversible modifications (*epigenetic modifications*), so RNA can experience a similar process (*epitranscriptomic modification*)—modifications that occur 'upon' (*epi-*) the set of RNA 'transcripts' (*transcriptome*).[1402] Such modifications are key to mRNA vaccine success.

The modifications replace native nucleosides (e.g., *uridine*) with chemically modified versions (e.g., *pseudouridine*). Certain chemical modifications have been more common in making nucleoside-modified mRNA. These include *5-methyluridine* (m5U), *2-thiouridine* (s2U), and *pseudouridine* (Ψ). The substitutions improve the stability of the mRNA, which as we've seen is necessary for it to be able to survive intact long enough to reach cytoplasmic ribosomes for translation.[1403] In this regard, too, substitutions with pseudouridine, s2U and N^6–methyladenosine (m^6A) all aid in suppressing host efforts to degrade the mRNA by RNases.[1404] Of those named above, pseudouridine, a naturally occurring modification derived from uridine and discovered in the 1950s, is perhaps the most well-known and often discussed. It impacts RNA structure, stability, and protein translation.[1405]

Now, let's set this general (and simplified) picture into another that enlarges what we are examining by considering the role of the modifications we have just mentioned. A different illustration may help (next).

[1402] See Kumar and Mohapatra, "Deciphering Epitranscriptome."
[1403] Jin et al., "Perspectives and Prospects on mRNA Vaccine Development," 3993.
[1404] Park et al., "mRNA Vaccines for COVID-19: What, Why, and How," 1449.
[1405] Morais, Adachi, and Yu, "The Critical Contribution of Pseudouridine," 2–4.

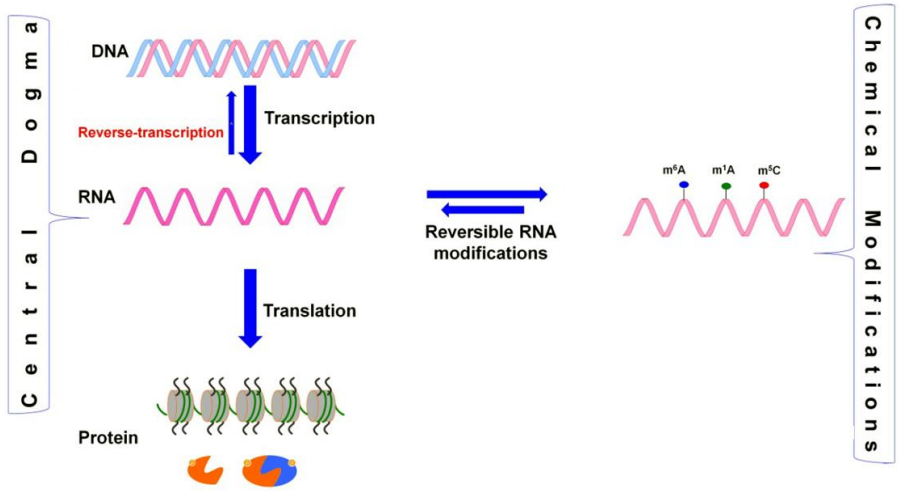


FIGURE 1 | [T]he dynamic RNA modifications [e.g., N^6-methyladenosine (m^6A), N^1-methyladenosine (m^1A), and N^6-hydroxymethyladenosine (hm^6A)] encrypt an additional layer of information and dynamically regulate the biological processes.

The illustration is extracted from the more complex one in Suresh Kumar and Trilochan Mohapatra. "Deciphering Epitranscriptome: Modification of mRNA Bases Provides a New Perspective for Post-transcriptional Regulation of Gene Expression." *Frontiers in Cell and Developmental Biology* 9 (Mar., 2021) art. 628415. doi.org/10.3389/fcell.2021.628415. The material appears on page 3 and is made available by Creative Commons Attribution (CC BY) License (https:// creativecommons.org/licenses /by/4.0/ legalcode). Changes have been made to font type in the illustration's explanatory text, which has been altered to exclude all text beyond the brief excerpt reproduced here focused on RNA modifications. Illustrative material above and below the RNA modifications, applied to DNA and histone proteins is also left aside, as are markings above and below the 'Reversible RNA modifications' shown here (and identical to it). The illustration of replication of DNA is also left aside. All changes are meant to simplify and concentrate focus on the topic at hand in this volume (and the reader is encouraged to examine the article figure and text).

We start with the Central Dogma (above, at left), which we first pictured in the illustration on the previous page. Now, though, we have added the idea of reversible RNA modifications. To this point, about 170 distinct modifications (mostly in tRNA and rRNA) have been identified thanks to advances in detection and RNA sequencing. Post-transcriptional base modifications in mRNA affect the sequence and structure of RNA, which in turn influence pre-mRNA splicing, mRNA export, translation, cellular localization, and degradation (longevity). Thus modifications serve as self-regulatory mechanisms.[1406]

At the right in the preceding illustration—'Chemical Modifications'—are pictured three nucleosides with base modifications: N^6–methyl-adenosine (m^6A), N^1-methyladenosine (m^1A), and N^6-hydroxymethyladenosine (hm^6A). These aren't the only such nucleosides with base modifications. Others include

[1406] Kumar and Mohapatra, ""Deciphering Epitranscriptome," 2, and Harcourt, Kietrys, and Kool, "Chemical and Structural Effects of Base Modifications in Messenger RNA."

N^4-acetylcytidine (ac4c), inosine, pseudouridine, 5-methylcytidine (m^5C), and 5-hydroxy-methylcytidine (hm^5C). A brief overview follows in the Table.[1407]

Name	Abbreviation	Known, Putative, or Hypothesized Effects
N^6–methyladenosine	(m^6A)	regulation of gene expression, splicing, stability, RNA degradation, regulation of levels of protein expression, etc.
N^1-methyladenosine	(m^1A)	interactions between RNA and protein, formation of alternative secondary RNA structures, etc.
N^6-hydroxymethyl-adenosine	(hm^6A)	potent and wide-ranging regulation functions in mRNA
N^4-acetylcytidine	(ac4c)	promotes mRNA stability; ac4c(+) mRNAs elevate translation efficiency,
Inosine	--	change amino acids encoded, affect local secondary structure, etc.
Pseudouridine	--	constrains flexibility in ssRNA, selective protein recognition, modu-lation of gene expression and mRNA stability (through *pseudouridylation*), etc.
5-methylcytidine	(m^5C)	unclear—possible structural effects
5-hydroxy-methylcytidine	(hm^5C)	affect epigenetic status of cell, influence translation of some mRNAs

This overview, as brief and incomplete as it is, may already venture us deeper in the pool than promised at the start of this book. But even as cursory as it is, the Table should serve to reinforce the basic idea that *base modifications make important differences in mRNA*—and being able to subtly change mRNA structure and/or function can have highly desirable effects for vaccine makers.

We noted earlier that nucleoside modifications enhance stability of the mRNA. Advances in modifying mRNA nucleosides also have improved mRNA translation. Another key has been the incorporation of pseudouridine and 1-methyl-pseudouridine to avoid detection by Toll-like receptors (TLRs 7 and 8) and other pattern recognition receptors.[1408] Both the Moderna (MR-NA-1273)

[1407] Table information based on a number of sources, including Arango et al., "Acetylation of Cytidine in mRNA"; Harcourt, Kietrys, and Kool, "Chemical and Structural Effects of Base Modifications in Messenger RNA"; Huber et al., "Formation and Abundance of 5-Hydroxymethylcytosine in RNA"; Ji et al., "N6-Methyladenosine in RNA and DNA"; Kumar and Mohapatra, ""Deciphering Epitranscriptome."

[1408] Saunders et al., "Lipid Nanoparticle Encapsulated Nucleoside-Modified mRNA Vaccines," 1.

and Pfizer (BNT162b2) vaccines encode the S protein of SARS-CoV-2 with modifications by two substitutions of viral amino acids (*prolines*) at residues 986 and 987 (986-KV-987 to 986-PP-987). These mutations, as noted earlier in the chapter, stabilize the S protein in a prefusion conformation that enhances immunogenicity—so much so that even at low doses the immunogenicity exceeds that of wild-type SARS-CoV-2.[1409] In short, modifying mRNA accomplishes a host of highly desirable features for vaccine developers.

Now, let's see what results from mRNA modifications. We will do this through more than one illustration. That below captures one aspect of what results—showing how base modifications affect gene regulation in various parts of a cell. This illustration captures some of what we are interested in here, notably what transpires in the cytoplasm (#s 4–7). But it also provides value in reminding us again that there is always a larger context to keep in mind.

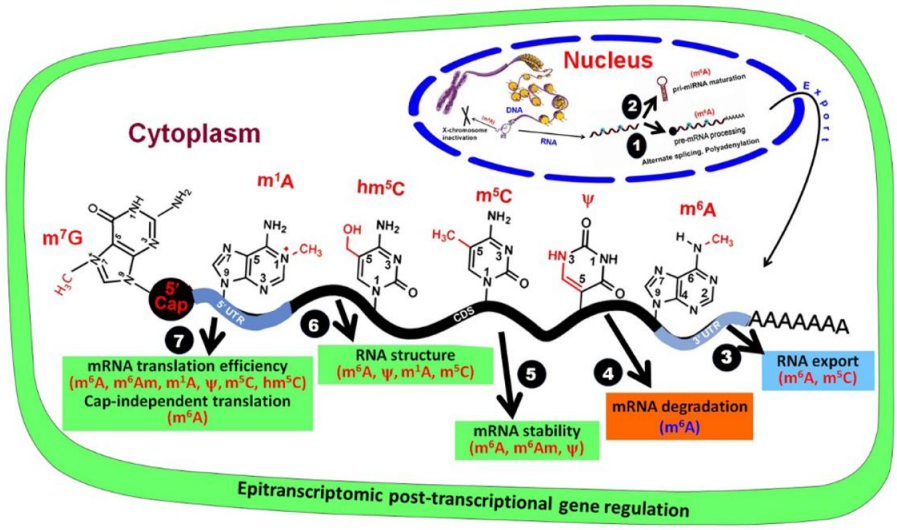


FIGURE 2 | Base modifications in mRNA affect post-transcriptional gene regulation. In the nucleus, RNA base modifications affect (1) pre-mRNA processing and (2) pri-miRNA maturation, and (3) their export from the nucleus. In the cytoplasm, RNA base modifications regulate (4) mRNA degradation, (5) mRNA stability, (6) RNA structure, and (7) mRNA translation efficiency.

The illustration is from Suresh Kumar and Trilochan Mohapatra. "Deciphering Epitranscriptome: Modification of mRNA Bases Provides a New Perspective for Post-transcriptional Regulation of Gene Expression." *Frontiers in Cell and Developmental Biology* 9 (Mar., 2021) art. 628415. doi.org/10.3389/fcell.2021.628415. The material appears on page 4 and is made available by Creative Commons Attribution (CC BY) License (https:// creativecommons.org/licenses /by/4.0/ legalcode). Changes have been made to font type in the illustration's explanatory text.

The bigger payoff comes in seeing how the resultant vaccine works in the body.

[1409] Jin et al., "Perspectives and Prospects on mRNA Vaccine Development," 3993.

The Inactive Ingredients

Based on what we have covered so far, it will come as no surprise that the active ingredient of mRNA requires some assistance. It is rendered by a rather sizable list of accompanying materials.

Inactive ingredients in the Moderna and Pfizer vaccines vary. They are: [1410]

Moderna mRNA-1273	*Pfizer* BNT162b2
<u>*Lipids*</u> PEG2000DMG: 1,2 -dimyristoyl-rac-glycerol, methoxypolyethylene glycol;	<u>*Lipids*</u> 2[(polyethylene glycol (PEG))-2000]-N,N-ditetradecylacetamide;
1,2-distearoyl-sn-glycero -3-phosphocholine;	1,2-distearoyl-sn-glycero-3-phosphocholine;
Cholesterol (Botani-Chol®);	Cholesterol (plant-derived);
SM-102: heptadecan-9-yl 8-((2-hydroxyethyl) (6-oxo-6-(undecyloxy) hexyl) amino) octanoate;	(4-hydroxybutyl) azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate);
<u>*Other ingredients*</u> Tromethamine; Tromethamine hydrochloride; Acetic acid; Sodium acetate; Sucrose	<u>*Other ingredients (salts* and sugars**)*</u> *Sodium chloride; *Monobasic potassium phosphate; *Potassium chloride; *Dibasic sodium phosphate dihydrate; **Sucrose

The *lipids* are part of the process used by the vaccine to assist the mRNA to enter human cells. The *other ingredients* are used from vaccine production through shipping and storage to preserve the vaccine until use.

Earlier, in our brief history, we encountered the early 21st century formation of a four compound lipid nanoparticle (LNP). Key to this success was the insight that adding *positively* charged lipids to liposomes (thus forming *cationic* liposomes) would balance negatively charged nucleic acids. Twin problems, though, accompanied this insight: such liposomes do not occur naturally, and they are toxic. But nanoparticle scientist Pieter Cullis, who had been working with liposomes since the early 1980s, found a solution that made for a vessel to transport nucleic acids that was neutral in blood.[1411] LNPs soon became a widely used way to transport mRNA and protect it from endosomal degradation.[1412]

[1410] CDC, "Frequently Asked Questions" (Sept. 3, 2021). Also see FDA, "Vaccine Information Fact Sheet."

[1411] Cross, "Without These Lipid Shells."

[1412] Himbert et al., "Erythro-VLPs," 2.

Two illustrations should help us better understand mRNA LNPs.

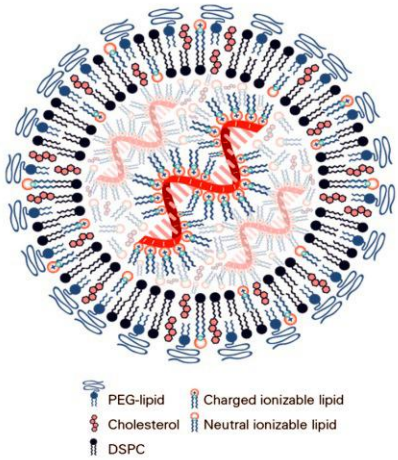


Figure 1. mRNA lipid nanoparticle structure. Recent studies using cryoelectron microscopy, small-angle neutron scattering and small-angle X-ray scattering have shown that the mRNA Lipid nanoparticle includes low copy numbers of mRNA (1–10) and that the mRNA is bound by the ionizable lipid that occupies the central core of the LNP. The polyethylene glycol (PEG) lipid forms the surface of the lipid nanoparticle (LNP), along with DSPC, which is bilayer forming. Cholesterol and the ionizable lipid in charged and uncharged forms can be distributed throughout the LNP. Structural schematics of other delivery systems are available in a recent review.

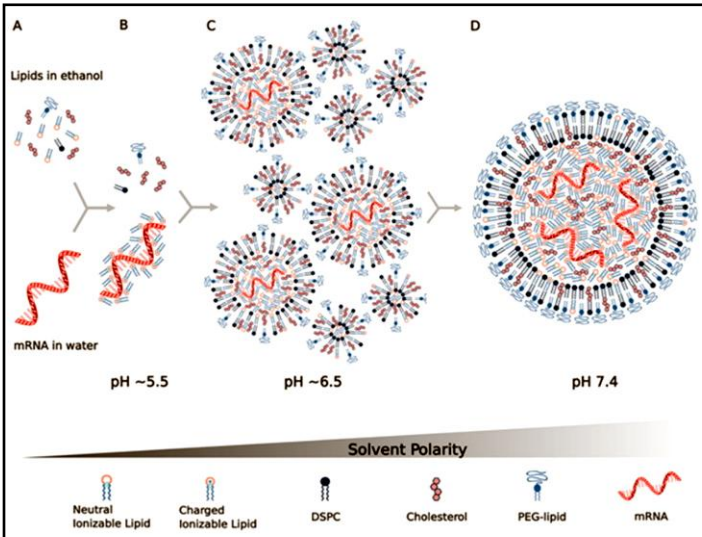


Figure 2. mRNA lipid nanoparticle assembly is achieved by (A) rapid mixing in a microfluidic or T-junction mixer of four lipids (ionizable lipid, DSPC, cholesterol, PEG–lipid) in ethanol with mRNA in an aqueous buffer near pH4. (B) When the ionizable lipid meets the aqueous phase it becomes protonated at a pH ~5.5, which is intermediate between the pKa of the buffer and that of the ionizable lipid. (C) The ionizable lipid then electrostatically binds the anionic phosphate backbone of the mRNA while it experiences hydrophobicity in the aqueous phase, driving vesicle formation and mRNA encapsulation. (D) After initial vesicle formation, the pH is raised by dilution, dialysis or filtration, which results in the neutralization of the ionizable lipid, rendering it more hydrophobic and thereby driving vesicles to fuse and causing the further sequestration of the ionizable lipid with mRNA into the interior of the solid lipid nanoparticles. The PEG–lipid content stops the fusion process by providing the LNP with a hydrophilic exterior, determining its thermodynamically stable size, and the bilayer forming DSPC is present just underneath this PEG–lipid layer.

The preceding two illustrations are from Michael D. Buschmann, Manuel J. Carrasco, Suman Alishetty, Mikell Paige, Mohamad Gabriel Alameh, and Drew Weissman. "Nanomaterial Delivery Systems for mRNA Vaccines." *Vaccines* 9 (2021) 65. https://doi.org/10.3390/vaccines9010065. The top one (Fig. 1) is material from page 6; the bottom one (Fig. 2) is from page 16. Changes have been made to both in the font type in the illustrations' explanatory text and in both the original figures have the drawings above the text. For Figure 1 the reference numbers to sources have been omitted. All material has been made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/ licenses/by/4.0/legalcode).

LNPs—sometimes referred to as *nanolipids*[1413]—join together four compounds into a LNP delivery system:[1414]

- *ionizable lipids* that have positive charges (at low pH) that bind to the negatively charged mRNA's backbone;
- *polyethylene glycol* (PEG-) *lipid*, a compound, added (*PEGylation*) to help stabilize the LNP, control its size, and protect it from immune system attack;
- *neutral phospholipids* to enhance LNP structural stability and enhance penetration of cell membranes; and,
- *cholesterol*, located on the surface of the LNP, to help stabilize the LNP, modulate delivery, and enhance penetration of cell membranes.

Besides the lipids involved in the delivery system there are other inactive ingredients, all of which contribute in some manner. The Moderna vaccine employs acids (*acetic acid*) and acid stabilizers (*tromethamine* and *tromethamine hydrochloride*), which help maintain vaccine stability. Both the Moderna and Pfizer vaccines contain *salts* to help balance acidity, thereby also contributing to vaccine stability, and *sugar* to assist vaccine integrity during freezing. Sugar (*sucrose*) also assists ongoing vaccine effectiveness by facilitating molecules retaining their shape throughout manufacturing, storage, distribution, and vaccination.

Many older vaccines use *adjuvants* (ingredients that enhance immune system response). One notable adjuvant is alum (aluminum oxyhydroxide), a substance that regularly draws anti-vax criticism and that is sometimes falsely claimed to be a part of the vaccines approved in the U.S. for COVID-19. But the mRNA vaccines not only do not have alum,[1415] they do not have any adjuvants. This is because *mRNA already exhibits self-adjuvating properties*, due to its capacity for binding to pattern-recognition receptors (PRRs, discussed in chapter 5).[1416]

[1413] See, for example, Cattel et al., "Vaccine Development and Technology," 879.

[1414] LNP delivery systems are succinctly described in Schoenmaker et al., "mRNA Lipid Nanoparticle COVID-19 Vaccines," 4–6. Also see Hou et al., "Lipid nanoparticles for mRNA Delivery."

[1415] Alum has been pointed out as lacking "the capability to promote the activation of $CD4^+$ and $CD8^+$ T cell responses, which has been demonstrated to coordinate with the antibody responses to provide protective immunity against the SARS-CoV-2" (Liang et al., "Adjuvants for Coronavirus Vaccines," 2).

[1416] Granados-Riveron and Aquino-Jarquin, "Engineering of the Current Nucleoside-Modified mRNA-LNP Vaccines," 1.

Conventionally, the vaccination process includes administration of the LNP delivery system by a one or two shot schedule. The injection is typically *intramuscular* (in upper arm muscles). However, some mRNA vaccines employ *intradermal* (between the layers of skin), or *subcutaneous* (under the skin) injection.[1417]

Our focus here is on the vaccine once within the body. Building upon our previous illustrations the process can be rather simply explained: "Upon intramuscular (IM) administration, the LNP system enables the uptake by host cells and the delivery of mRNA inside the cytosol, where the translation of the mRNA sequence into the S protein occurs in the ribosomes."[1418]

Let's start with an illustration of the biology of mRNA vaccines and to help make sense of it we will set it alongside DNA vaccines.

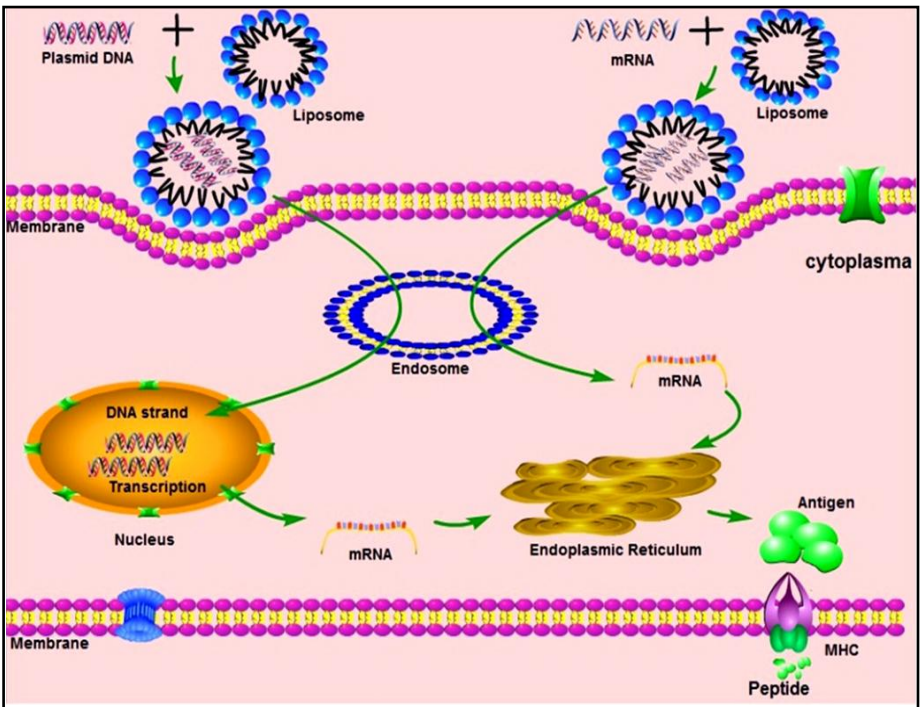


FIGURE 1 | The mechanisms of different nucleic acid vaccines, including DNA vaccines, mRNA vaccines. MHC, Major histocompatibility complex.

The illustration is from Cuiling Zhang, Giulietta Maruggi, Hu Shan, and Junwei Li. "Advances in mRNA Vaccines for Infectious Diseases." *Frontiers in Immunology* 10 (Mar., 2019) art. 594. doi: 10.3389/fimmu.2019.00594. Material is from page 3 and is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/legalcode). Changes have been made to font type in the illustration's explanatory text.

[1417] Zhang et al., "Advances in mRNA Vaccines," 5.
[1418] Schoenmaker et al., "mRNA Lipid Nanoparticle COVID-19 Vaccines," 1.

Next we can look at an overview specific to an mRNA vaccine.

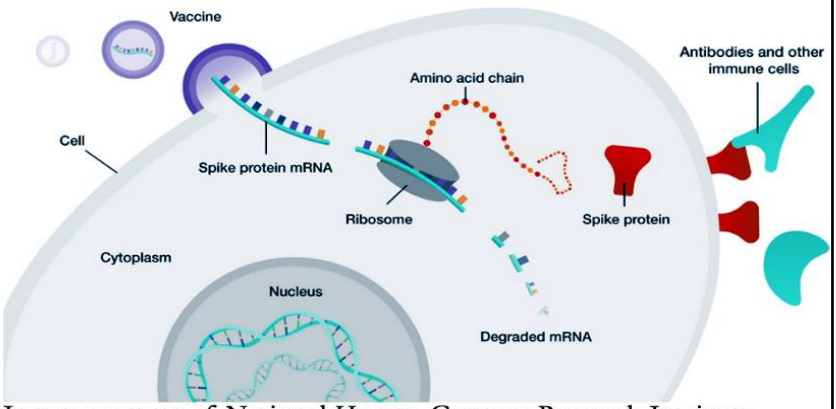


Image courtesy of National Human Genome Research Institute (https://www.genome.gov)

Finally, our last illustration offers more detail with the sequence in order.

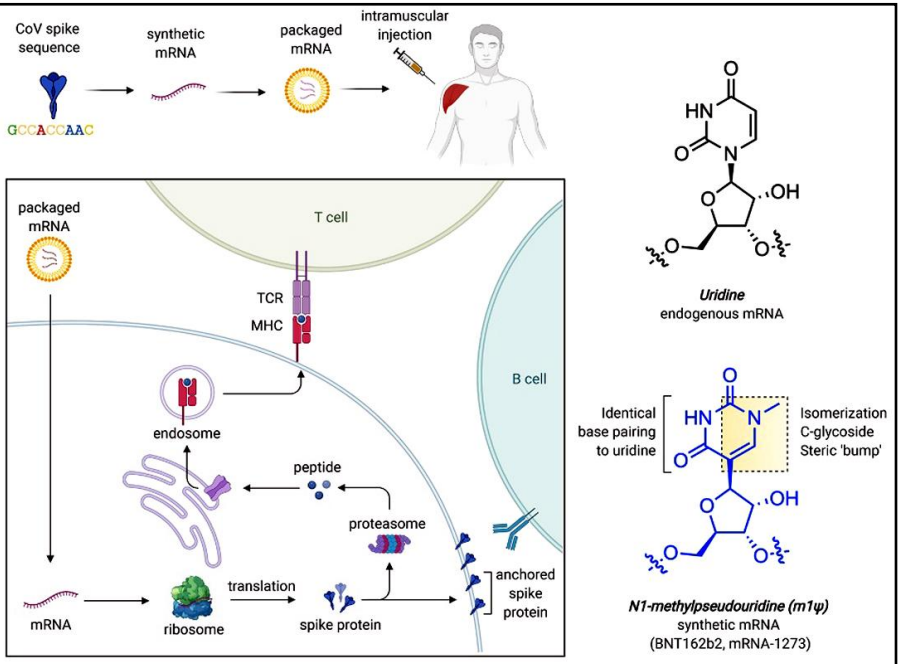


Figure 1. (a) mRNA-based COVID-19 vaccine strategy. (b) Structural features of uridine and m1Ψ. TCR = T-cell receptor. MHC = major histocompatibility complex.

Figure in Kellie D. Nance and Jordan L. Meier. "Modifications in an Emergency: The Role of N1-Methylpseudourodine in COVID-19 Vaccines." *ACS Cent. Sci.* 7 (2021) 748–56. https://doi.org/10.1021/acscentsci.1c00197. Public domain ("Not subject to U.S. copyright").

The mRNA lipid nanoparticle (LNP) delivery system introduces the mRNA into cells in a process termed *transfection* (literally, 'to put into')—a nonviral way of getting material into cells. The LNPs enter cells by *endocytosis*—a way to take in large molecules from outside the cell and then break them down inside the cell (covered in chapter 2), thus forming an *endosome* (a transport system within the cell) without destroying the cell membrane. Three different kinds of cells in the vaccinated host can be transfected:

- *non-immune cells* (e.g., muscle and epidermal cells) at the injection site;
- *tissue-resident immune cells* at the injection site (e.g., antigen-presenting cells (APCs) like dendritic cells and macrophages); and
- *immune cells in peripheral lymphoid organs* after transfer through the lymphatic system to adjacent lymph nodes or the spleen.

The endosome is directed toward *lysosomes* (specialized structures with digestive enzymes) for degradation, but ionizable lipids in the LNP enable endosomal escape, thus preserving structural integrity. Chemical changes in the endosome result in the mRNA's delivery into the cytoplasm, where ribosomes translate its message into the desired protein.[1419]

In the real world of the human body (i.e., *in vivo*—'in what is living'), LNPs must overcome barriers outside target cells (*extra*cellular) and inside them (*intra*-cellular). These include:

- First, the transported mRNA must be protected from *nuclease degradation* (i.e., breakdown of the mRNA by an enzyme called a 'nuclease') in physiological fluids.
- Second, the LNP needs to evade interception by the *mononuclear phagocyte system* (MPS), which includes blood monocytes and tissue macrophages, and clearance by *renal glomerular filtration* (i.e., cleansing by the kidneys) after systemic administration.
- Third, the LNP delivery system needs to reach the target tissues, followed by internalization by the target cells.
- Finally, the mRNA must escape endosomes to reach the cytoplasm (the cell area outside the nucleus), where its translation by ribosomes into the desired protein (the S protein) occurs.[1420]

The LNP has successfully completed its job when its mRNA package is delivered in the cytoplasm for action. There occurs genomic sequences release targeting both ribosomal machinery and the rough endoplasmic reticulum.[1421] Translation of the mRNA generates production of the S protein to goad an immune response. The following two illustrations complete the picture.

[1419] Fang et al., "Advances in COVID-19 mRNA Vaccine Development," 1–2.
[1420] Hou et al., "Lipid Nanoparticles for mRNA Delivery," 1084.
[1421] Tsiambas et al., "Impact of Ribosome Activity," 4.

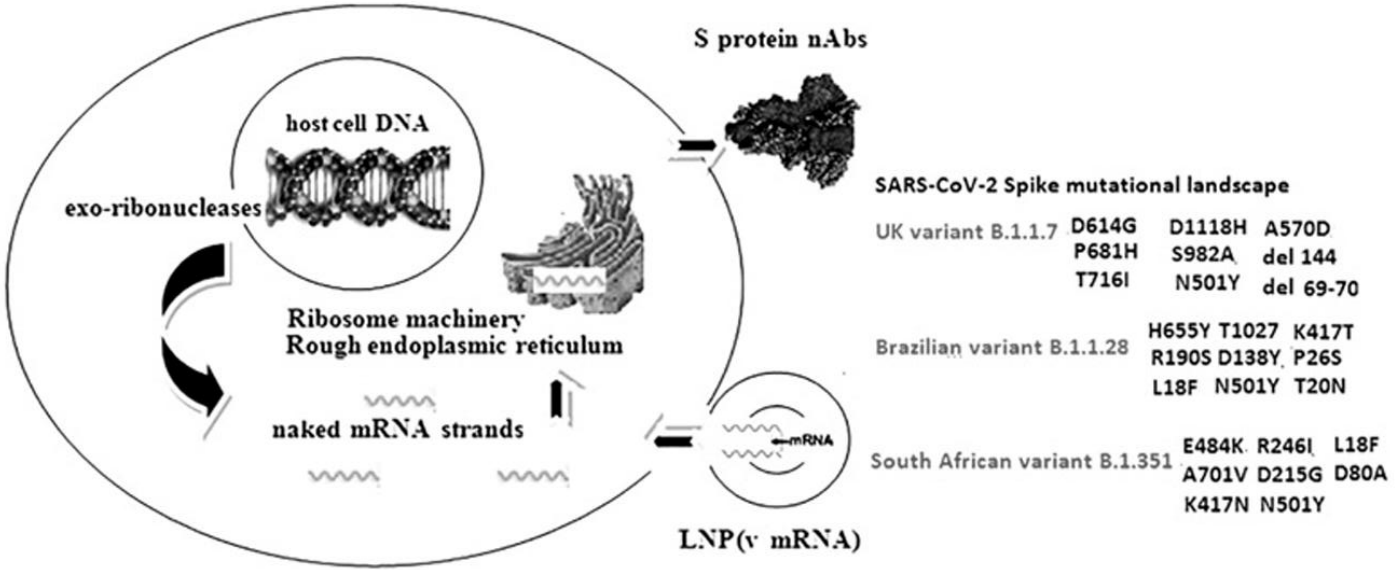


FIGURE 1 | Schematic presentation of a target cell—mRNA LNP based vaccine interaction and the SARS-CoV-2 Spike glycoprotein major mutational landscape. Nucleoside-modified viral messenger RNA (v mRNA) genomic sequence is encapsulated within nanoparticles—specifically lipid ones (LNPs). Insertion of the LNP-m RNA complex is followed by translation of the corresponding transcript in the ribosome machinery and final synthesis of the S protein S protein neutralizing antibodies (nAbs) in rough endoplasmic reticulum which are exported from the cell membrane in order to sensitize T cells and motivate a specific anti SARS-CoV-2 immune response. Mutations (specific RNA substitutions and deletions) affecting the spike glycoprotein create the corresponding virus RNA variants and are responsible for different rates of anti-SARS-CoV-2 resistance reducing immune response in the first generation v-mRNA vaccine.

The illustration is from Evangelos Tsiambas, Aristeidis Chrysovergis, Vasileios Papanikolaou, Nicholas Mastronikolis, et al., "Impact of Ribosome Activity on SARS=CoV-2 LNP-Based mRNA Vaccines." *Frontiers in Molecular Biosciences* 8 (Apr., 2021) art. 654866. doi: 10.3389/fomlb.2021.654866. Material is from page 3 and is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/legalcode). Changes have been made to font type in the illustration's explanatory text.

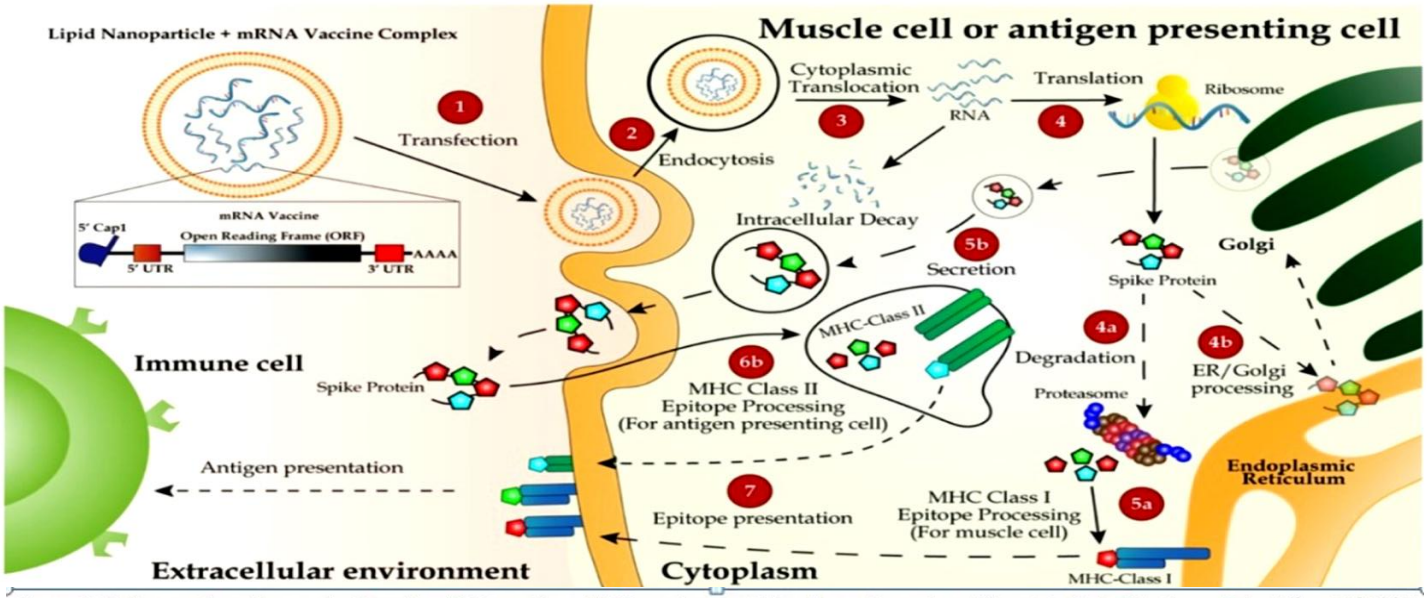


Figure 2. Delivery and working mechanism of a mRNA vaccine. mRNA vaccine, containing the coding region of S protein flanked by the optimized 5'- and 3'-UTRs and polyA tail, is synthesized via IVT, followed by 5'-capping with a 5'-cap analogy and encapsulation with LNP for IM injection (step 1). The vaccine is delivered into muscle cells or antigen-presenting cells such as dendritic cells or macrophages via endocytosis (step 2). mRNA molecules are unloaded from LNPs and translated to S protein in the ribosome (step 3). Newly synthesized S protein is secreted to extracellular space, internalized via endocytosis into antigen-presenting cells and incorporated as a part of MHC class II antigen presentation complex (steps 5b, 6b, and 7) to present the antigen to immune cells including T and B cells. Partially degraded S peptides by proteosomes are incorporated into MHC class I complexes, which are then transported to plasma membranes and also presented as antigens to immune cells (steps 4a, 4b, 5a, and 7).

Illustration is from Jung Woo Park, Philip N.P. Lagniton, Yu Liu, and Ren-He Xu. "mRNA Vaccines for COVID-19: What, Why, and How." *International Journal of Biological Sciences* 17/6 (2021) 1446–60. doi: 10.7150/ijbs.59233. Material appears on page 1453 and is made available by Creative Commons Attibution (CC BY) License (https://creativecommons.org/licenses/by/4.0/ legalcode). The original has been changed in font size and type for explanatory material and a source number [132] has been deleted (after B cells).

Obviously, once delivered and given adequate time to work, a vaccine must be evaluated as to its effectiveness—how well it works in the real world of human bodies. Let's look at this in context.

The delivered mRNA message to the vaccinated cells instructs it to build a protein—the Spike (S) protein of SARS-CoV-2. Now this may seem a foolhardy thing to do, like instructing a naive child how to build a bomb. But a more apt analogy would be instructing the child to build a nonfunctional model of a bomb's casing in order to learn firsthand what it looks like and thereafter act appropriately if encountering such an object. In this case, the S protein is without the rest of the virus and its machinery. It cannot replicate and it is also fixed in one position. So the immune system is taught to recognize the danger without the actual hazards presented by the virus being present.

Another way to look at this process is like a coach using a practice squad to present to the team what the next opponent's game plan looks like. In this way his or her team can practice—scrimmage—against the opponent's tactics before actually facing them when a defeat would be devastating. Similarly, military commanders prepare their forces with so-called war games—a way of getting the troops ready for actual combat without the stakes being so high. That is what vaccines do.

Gauging how effective the instruction has been can be done by measuring the immune system response long before any actual infection occurs. Since the goal is to prod the immune system into making an antibody to fight against the S protein, if the immune system does so robustly, then the vaccine can be said to be working at a biological level. This takes time and is measured over time.

Early in the pandemic a study was done with 59 health care workers who had been enrolled in a hospital serosurvey study (such studies were discussed in an earlier chapter). The volunteers were sorted into three groups based on serology results (antibody-negative; antibody positive without symptoms; antibody positive with symptoms). Then all the volunteers received an initial dose of mRNA vaccine. The researchers found that *even a single dose of either the Pfizer or Moderna vaccine resulted in higher antibody titers and higher neutralization titers at two weeks post-vaccine for the antibody positive groups than for those antibody-negative*. These results provide evidence of a good immune response prompted by vaccine.[1422]

The adaptive immune system after application of mRNA vaccine has been observed to begin providing protection *as quickly as 10–12 days after the first dose*. Moreover, this observed protection includes spike (S) protein-specific antibodies, virus-specific $CD4^+$ T_H1 cells, and IFN-γ-producing $CD8^+$ T-cells. When the vaccination course is completed, neutralizing antibodies (NAbs) become evident, too.[1423] All these indicators say that the vaccine is doing its job.

But how does that job compare to what the virus does in natural infection?

[1422] Saadat et al., "Binding and Neutralization Titers After a Single Vaccine Dose."
[1423] Oberhardt et al., "Rapid and Stable Mobilization," 268.

A successful vaccine has to imitate its opponent enough to help the immune system learn an effective defense against the real opponent. So let's compare how the virus and vaccine act.

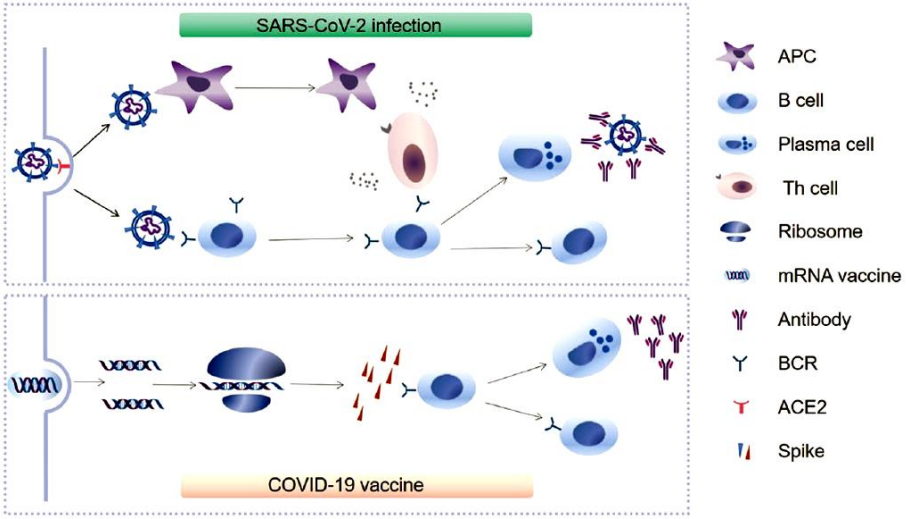


Fig. 1 Proposed models of virus-specific humoral immunity induced by SARS-CoV-2 infection or an mRNA vaccine. (1) SARS-CoV-2 infection: SARS-CoV-2 invades host cells by both endocytosis and membrane fusion for viral entry through binding of the viral spike (S) glycoprotein to the viral receptor ACE2. Next, SARS-CoV-2 is processed by APCs, exposing its hidden epitopes. Subsequently, APCs present viral antigens to Th cells, followed by activation and lymphokine secretion and B cell activation; viral antigens may also directly stimulate B cells. Under stimulation with viral antigens, the majority of B cells proliferate and differentiate into plasma cells to produce specific antibodies against SARS-CoV-2. Simultaneously, a small proportion of B cells develop into SARS-CoV-2-specific memory B cells. Upon SARS-CoV-2 reinfection, the virus-specific memory B cells can be rapidly transformed into plasma cells to produce specific antibodies. The specific antibodies (primarily neutralizing antibodies) produced by plasma cells can neutralize SARS-CoV-2 and block the interaction between the spike protein and ACE2. (2) SARS-CoV-2 vaccine: after inoculation, an mRNA vaccine encoding the relevant S protein encapsulated by lipid nanoparticles enters a cell, and the S protein is synthesized by ribosomes. Subsequently, B cells are stimulated by the S protein to proliferate and differentiate into specific plasma and memory B cells. Finally, the plasma cells produce large amounts of specific antibodies against SARS-CoV-2, which may play a protective role in viral reinfection. ACE2 angiotensin-converting enzyme 2, APC antigen-presenting cell, BCR B cell receptor, SARS-CoV-2 severe acute respiratory syndrome coronavirus 2, Th cells T helper cells.

Illustration from Jiaxin Zheng, Yingying Deng, Zhenyu Zhao, Binli Mao, Mengji Lu, Yong Lin, and Ailong Huang. "Characterization of SARS-CoV-2 Specific Humoral Immunity and Its Potential Applications and Therapeutic Prospects." *Cellular and Molecular Immunology* 19 (2022) 150–57. https://doi.org/10.1038/s41423-021-00774-w. Material is from page 151 and is made available by Creative Commons Attribution (CC BY) License (https:// creativecommons.org/licenses /by/4.0/ legalcode). Changes have been made to font type in the illustration's explanatory text.

Safety & Efficacy of mRNA Vaccines

As we saw in the previous chapter, vaccines are judged by efficacy (or effectiveness) and by safety (i.e., absence of adverse events after vaccination). To win authorization for use a vaccine must satisfactorily establish both. Even before either approved mRNA vaccine for COVID-19 appeared, the mRNA technology's safety was recognized as one of its beneficial features over other vaccine platforms (e.g., DNA, subunit, killed and live attenuated virus). This is because as a non-infectious, non-integrating platform, there is no potential risk of infection or insertional mutagenesis. Similarly, its efficacy was touted because various modifications make mRNA both highly stable and highly translatable.[1424]

Safety

Although we will examine both efficacy and safety for each of the two approved mRNA vaccines, let us first start with some comparative information on safety. What is placed here reflects information obtained from the vaccine trials, i.e., *before* the vaccines were authorized for use. This information is important because it helps explain *why* the vaccines were approved. They had to demonstrate safety and as part of that a record of adverse events was kept. The Table (right) summarizes such data for both Moderna and Pfizer mRNA vaccines.

The Table at right is excerpted from Table 1 in Yingqui Jin, Chen Hou, Yonghao Li, Kang Zheng, and Chuan Weng. "mRNA Vaccine: How to Meet the Challenge of COVID-19." *Frontiers of Immunology* 12 (Jan., 2022) art. 821538. doi: 10.3389/fimmu.2021.821538. The material appears on page 5 and is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/ legal code). Changes have been made by excerpting only the information pertaining to the two vaccines covered in this book. The Table also provides information on four other vaccines in trials.

TABLE 1 | The common adverse reactions of mRNA vaccine candidates in vaccine trials.

Vaccine name	BNT162b2 (26, 69)	mRNA-1273 (25, 76)
Phase	III/IV	IV
Developers	BioNTech/Fosun Pharma/ Pfizer	Moderna/NIAID
Age at vaccination	≥16 years	≥18 years
Route of administration	IM	IM
Number of doses	2	2
Duration	The second injection was given after 28 days	The second injection was given after 28 days
Time of adverse reaction	Mostly start about 15 min	Mostly start about 15 h after vaccination
	Right axillary lymphadenopathy; paroxysmal ventricular arrhythmia; right leg paresthesia	Pneumonia; immediate systemic allergic reactions
Adverse reaction	The injection site: mild-to-moderate injection site pain Respiratory system: cough; shortness of breath Digestive system: decrease appetite; nausea, vomiting; diarrhea Nervous system: fatigue; lethargy; paralysis; headache; dizziness Systemic reaction: asthenia, malaise Skin: night sweats, hyperhidrosis	

Phase: a stage in the development of mRNA vaccine clinical trials. IM, intramuscular injection.

[1424] Pardi et al., "mRNA Vaccines," 261.

Looking at the listed adverse reactions can be alarming. Certainly no one wants to add to the discomfort of a shot further negative effects. Since we ered this matter in the previous chapter, here let us note only a few things. First, the likelihood of adverse events from vaccination is far less than from ing COVID-19, while the likelihood of contracting COVID-19 with its symptoms is far greater without vaccination. As some would say, 'Pick your poison.' As we have seen, vaccines *must* mimic the virus while avoiding doing the same degree of damage. But that doesn't mean kicking the immune system into action won't be a little painful. Second, the overwhelming majority of adverse events are minor. As the authors of the study from which the above Table comes observe, mild to moderate side effects are an *expected* outcome that demonstrate the vaccine has gotten the body's attention and provoked an immune response. Many so-called 'allergic' reactions fit within the expected range of reactions. Genuine allergic reactions typically occur within the first 30 minutes after injection and are usually related to inactive vaccine ingredients (*incipients*). Third, as the same authors go on to note, adverse reactions appear to vary among vaccine platforms and specific vaccines. In general, serious side effects (e.g., serious metabolic, immune system, musculoskeletal, renal disorders, serious gastrointestinal complications, thrombosis, and infections) are all seen *less* in mRNA vaccines than in other kinds (and far less than in COVID-19).[1425]

Efficacy

Efficacy and efficiency—two terms that can be distinguished from one another but generally are not—refer to how well a vaccine actually works. As we have seen the primary goal is to prevent infection, and thus interrupt transmission. But we have also seen how difficult it is to meet this primary goal and so an important secondary goal is to limit the degree of whatever infection might occur. Vaccine makers are content enough if meeting the first goal happens at least half the time (50% efficacy)—in other words, if taking the vaccine cuts in half the likelihood of getting infected. In the United States the Food and Drug Administration (FDA) set that as the bar that had to be surpassed. In actuality, the approved mRNA vaccines far exceeded that standard (see below).

Vaccine efficacy is related to success in provoking a robust immune response (*immunogenicity*). Lipid nanoparticle (LNP) nucleoside-modified mRNA vaccines possess potent immunogenicity for viral proteins. In part this is because the LNP delivery system gets the mRNA to antigen-presenting cells (APCs) which quickly activate naïve T-cells. Additionally, the vaccine produces strong antigen-specific germinal center (GC) B-cell and T-cell responses. Follicular T-cells assist B-cells in GCs as they undergo affinity maturation, making the resulting antibodies highly prepared to respond to the viral S protein.[1426]

[1425] Jin et al., "mRNA Vaccine," 4. For more details, see Bolich, *Understanding COVID-19 Vaccine Arguments*, 67–75.

[1426] Saunders et al., "Lipid Nanoparticle Encapsulated Nucleoside-Modified mRNA Vaccines," 9.

Moderna (mRNA1273)

Moderna's vaccine received emergency use authorization (EUA) on December 18, 2020 (a week after Pfizer's). Although it effectively bars the virus in many instances, it cannot guarantee stopping infection—though it actually does that with astounding success. What it also does spectacularly well is minimize the effects of infection in the unlikely event that it should occur. It does so by helping the immune system to be armed and ready.

Specifically, the vaccine induces the immune system to produce *neutralizing antibodies* (NAbs) to act against the spike (S) protein of SARS-CoV-2. As we have seen, such antibodies block infection. Additionally, the vaccine induces production of other binding antibodies—*non-neutralizing antibodies* (n-NAbs). These antibodies, though they cannot stop infection, act to combat it vigorously should it happen. This is important because as SARS-CoV-2 has mutated its variants have become more successful against NAbs—but not against n-NAbs. The latter rely on Fc-FcR and Fc-Complement interactions to destroy infected cells and thus stop the spread of infection. Studies indicate that n-NAb functions such as these continue to be very effective even when variants experience more success against NAbs. This has been seen with Moderna's vaccine.[1427]

Numerous studies confirmed the Moderna vaccine's efficacy and safety before it won approval. Here we shall examine only a few research studies—simply picking voices out of a large and loud chorus of agreement.

In late 2020, an article in *The New England Journal of Medicine* reported on a study of some 40 older adults receiving the Moderna vaccine (100 μg dose). They experienced mainly mild or moderate adverse events, with no serious events reported. The most common adverse events were injection site pain, chills, headache, fatigue, or myalgia (muscle aches and pain). These typically occurred within a day of vaccination. With respect to efficacy against the virus, the vaccine displayed robust virus-neutralizing activity, which persisted a month after vaccination. The study noted, "The mRNA-1273 vaccine induced high levels of both binding and neutralizing antibodies in older adults, and the time- and dose-dependent trends were similar to responses in younger adults."[1428]

A Kaiser Permanente Southern California (KPSC) health system study reported in late 2021—an interim analysis as part of a planned five year observational study—examined vaccine effectiveness for 352,878 recipients of the Moderna vaccine against a comparable matched group of 352,878 unvaccinated individuals. There were relatively more females (59.4%) and relatively more non-Hispanic Whites (38.7% compared to 32.4% Hispanic). During follow-up through June 30, 2021, COVID-19 infections occurred in both groups, but dramatically more among the unvaccinated. Here are some comparisons:

[1427] Kaplonek et al., "mRNA-1273 Vaccine-Induced Antibodies," 356.

[1428] Anderson et al., "Safety and Immunogenecity of SARS-CoV-2 mRNA-1273 Vaccine." The quote is from p. 2433.

COMPARING OUTCOMES	*Vaccinated* Incident rate per 1,000 (Cases—actual number)	*Unvaccinated* Incident rate per 1,000 (Cases—actual number)
COVID-19 infections	2.77 (289)	20.20 (1,144)
Hospitalizations	0.12	3.21
Deaths	0.01	0.44[1429]

The measured effectiveness of the Moderna vaccine for up to five months was as follows:

- 87.4% against infection
- 95.8% against hospitalization
- 97.9% against hospitalized death[1430]

The study also concluded that among those who have experienced COVID-19 vaccination with Moderna may offer additional protection beyond whatever may be conferred by natural immunity.[1431]

The rise of the Omicron variant to dominance in the United States in late 2021 raised significant concern over the effectiveness of the authorized vaccines. Moderna released news on December 20, 2021, reporting that its authorized booster shot (50 μg) increased neutralizing antibody levels against Omicron 37-fold (and 83-fold with a dose of 100 μg). Not only did the booster demonstrate effectiveness, but with respect to safety Moderna reported that adverse events following the authorized booster shot were, after 7 days, generally comparable to those seen after the two-dose vaccination schedule.[1432] As detailed a little further below, compared to AstraZeneca, Johnson & Johnson, and Pfizer vaccines, Moderna's vaccine induced not only the highest levels of binding antibodies but also the highest and most enduring SARS-CoV-2-specific T-cell responses.[1433]

Two large-scale reports published in 2022 can round out this brief look at the scientific literature. The first, which included data on 2,709,899 persons within 14–120 days after full vaccination, estimated Moderna's effectiveness against severe disease at 96% (93–98%).[1434] In the second, a meta-analysis and systematic review of 51 studies found that Moderna's vaccine achieved 98.1% efficacy—the highest among the three studied (the others were Pfizer (91.2%) and CoronaVac (65.7%), which is not an mRNA vaccine but an inactivated whole virus vaccine).[1435] Clearly Moderna's vaccine succeeds.

[1429] Bruxvoort et al., "Real-World Effectiveness of the mRNA-1273 Vaccine."
[1430] Bruxvoort et al., "Real-World Effectiveness of the mRNA-1273 Vaccine," 7.
[1431] Bruxvoort et al., "Real-World Effectiveness of the mRNA-1273 Vaccine," 12.
[1432] Moderna, "Moderna Announces Preliminary Booster Data."
[1433] GeurtsvanKessel et al. "Divergent SARS-CoV-2 Omicron-Specific . . . ," 8.
[1434] Premikha et al., "Comparative Effectiveness," 3.
[1435] Zheng et al., "Real-World Effectiveness of COVID-19 Vaccines," 256.

Pfizer/BioNTech (BNT162b2)

Of the three approved COVID-19 vaccines in the United States, the Pfizer vaccine COVID-19 proved the most used from the start of distribution through the first half of 2022. By the end of the first week of June, 2022, Pfizer's vaccine had been administered in 347,501,597 doses to Americans—more than 60% of all vaccine doses in the pandemic.[1436] It received emergency use authorization (EUA) December 11, 2020, and full approval August 23, 2021.

An early 2022 published report on the Pfizer vaccine found it first induces very robust, though relatively short-lived blood cells that divide rapidly, secrete antibodies and bind to antigens (called a *transient circulating plasmablast*). At the same time, the germinal center (GC)—which we saw in a preceding chapter produces activated B-cells that may serve as either memory B-cells or effector long-lived plasma cells—shows a persistent response. This research found that two doses of the Pfizer vaccine resulted in antibodies that could bind the S protein and that robust production of such antibody cells by the GC persisted at least 29 weeks (7+ months).[1437]

But even a single dose of vaccine—half the initially recommended course for mRNA vaccines—produces significant benefits. A study found that while the power of the antibodies to neutralize the virus should it be encountered—its *neutralization potency*—was forming in the body, other antibody functions, such as Fc-mediated effector functions, might be contributing to vaccine efficacy early on. That research reported that a single vaccine dose administered to those who had not been previously infected ('SARS-CoV-2 naive individuals') brought their SARS-CoV-2-specific humoral and T-cell responses to levels like those found in infected individuals some nine months earlier.[1438]

In Israel, the vaccination of adolescents coincided with the Delta wave of the pandemic. Research in that nation reported Pfizer's effectiveness with this population to be 91.5% against SARS-CoV-2 infection. In a comparable period of time, none of the vaccinated teenagers required hospitalization for COVID-19; of the unvaccinated who tested positive, 33 of 9,969 (0.33%) did require hospitalization.[1439]

Studies on Pfizer's efficacy against the emerging Omicron variant were mixed. It performed relatively well in laboratory tests with Pfizer reporting that after a course of vaccination plus booster shot of its vaccine people showed neutralizing antibody levels against Omicron comparable to two dose levels against other variants.[1440] In other words, though, that meant it took three shots to achieve the vaccine efficiency against Omicron that two shots achieved against earlier variants.

[1436] CDC, "COVID Data Tracker" (June 6, 2022; https://covid.cdc.gov/covid-data-tracker/#vaccinations_vacc-total-admin-rate-total.)

[1437] Kim et al., "Germinal Centre-Driven Maturation of B Cell Response," 1–3.

[1438] Tauzin et al., "A Single Dose of the SARS-CoV-2 Vaccine BNT162b2," 1146–47.

[1439] Glatman-Freedman et al., "Effectiveness of BNT162b2," 2920–21.

[1440] Calloway, "Omicron," 368.

Research also has shown that the Pfizer vaccine reactivates preexisting spike S-II cross-reactive CD4+ T-cells (i.e., T-cells produced from previous exposure to a different human coronavirus).[1441]

Research Article Summary
(*Accompanying Illustration*)

Cross-reactive CD4+T cells enhance SARS-CoV-2 immune responses upon infection and vaccination.(A) Preexisting SARS-CoV-2 S-II–and spike (S)816-830–cross-reactive T cells show booster response characteristics upon SARS-CoV-2 infection and vaccination and their abundance correlates with higher protective anti–SARS-CoV-2 S1-IgG titers and higher functional T cell avidity. (B) The frequency of HCoV-reactive and SARS-CoV-2–cross-reactive T cells decreases with age.

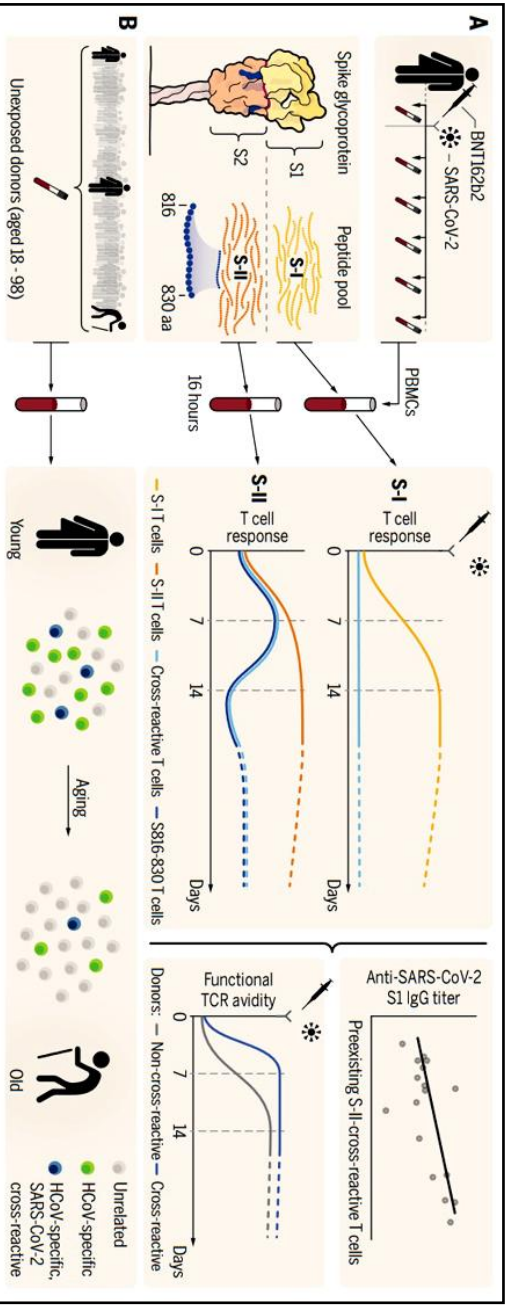


Illustration from Research Article Summary in Lucie Loyal, Julian Braun, Larissa Henze, Beate Kruse, et al. "Cross-Reactive CD4+ T Cells Enhance SARS-CoV-2 Immune Responses Upon Infection and Vaccination." *Science* 374 (2021) 171. Full article identifier: eabh1823. DOI: 10.1126/science.abh 1823. Material is made available by Creative Commons Attribution (CC BY) License (https:// creativecommons.org /licenses/by/4.0/ legalcode).

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[1441] Loyal et al., "Cross-reactive CD4+ T Cells Enhance SARS-CoV-2 Immune Responses," 5.

Midway through 2022, many in the field of vaccinology were expressing optimism over the development and use of *self-amplifying RNA* (saRNA). This kind of vaccine use enzymes (*replicons*) from alphaviruses (small, spherical, enveloped viruses with single-stranded, positive-sense, RNA genomes). This added material prompts repeated copying of the mRNA, keeping it active longer. The attractiveness of this includes needing only a single, smaller dose of vaccine.

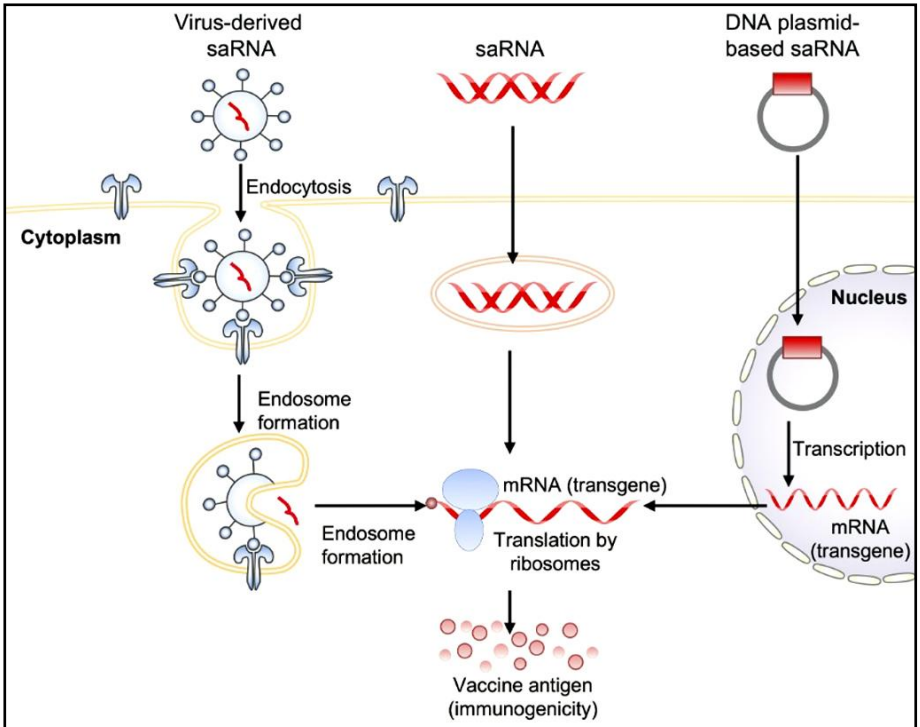

FIGURE 3 | Obtaining antigen expression by self-amplifying RNA vaccination. Three routes for the delivery of self-amplifying RNA (saRNA) are shown. These include: 1) virus-like RNA particles that deliver saRNA to the cytoplasm by receptor-mediated endocytosis, 2) direct delivery of in vitro transcribed replicon saRNA to cells either in saline or in synthetic formulations, and 3) plasmid DNA carrying replicase genes and the transgene into the nucleus, where it is transcribed, generating replicon saRNA, which is then transported to the cytoplasm. The three saRNA vaccination routes finally generate messenger RNA (mRNA), which is translated via ribosomes to produce vaccine antigen.

The illustration is from SangJoon Lee and Jin-Hyeob Ryu. "Influenza Viruses: Innate Immunity and mRNA Vaccines." *Frontiers in Immunology* 12 (Aug., 2021) art. 710647. doi: 10.3389/fimmu.2021.710647. Material is from page 7 and is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/legalcode). Changes have been made to font type in the illustration's explanatory text.

A comparative glance at the genetic sequencing of conventional mRNA and saRNA vaccines is revealing, too:

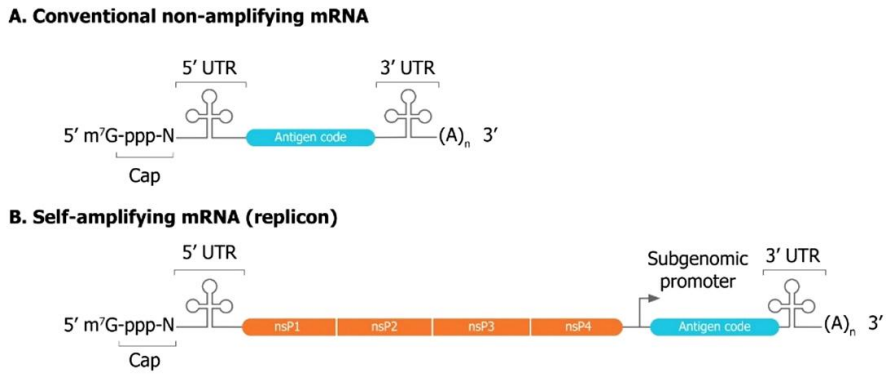

Figure 4. A comparison of mRNA vectors. Both conventional (A) and self-amplifying (B) mRNAs share basic elements including a cap, 5' UTR, 3' UTR, and poly(A) tail of variable length. Self-amplifying RNA (saRNA) also encode four non-structural proteins (nsP1–4) and a subgenomic promoter derived from the genome of the alphavirus. nsP1–4 encode a replicase responsible for amplification of the saRNA that enable lower doses than non-replicating mRNA.

The illustration is from SangJoon Lee and Jin-Hyeob Ryu. "Influenza Viruses: Innate Immunity and mRNA Vaccines." *Frontiers in Immunology* 12 (Aug., 2021) art. 710647. doi: 10.3389/fimmu.2021.710647. Material is from page 9 and is made available by Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0/legalcode). Changes have been made to font type in the illustration's explanatory text.

A *lipid nanoparticle* (LNP) *encapsulated self-amplifying RNA* (saRNA) vaccine varies from approved 'conventional' mRNA vaccines in a number of significant ways. For example, ssRNA vaccines are based on the genome of positive strand viruses (typically alphaviruses). The structural protein sequences are replaced with the gene of interest (the resulting genomes are referred as *replicons*). They focus on an engineered viral genome containing the genes encoding for RNA replication—hence the 'self-amplifying' aspect of the vaccine. The key to their success is this ability to 'self-amplify—that is, self-replicate when in the cytoplasm of target cells. This mimics what SARS-CoV-2 does and the multiple copies of the antigen presented thus prompts action by both the innate and adaptive immune systems.[1442] The ssRNA vaccines also are different in not containing modified-nucleosides (which might interfere with replication).[1443] Like the approved mRNA vaccines, saRNA vaccines do not require an adjuvant to boost immunogenicity; the LNP is sufficient in activating an immune response.[1444]

[1442] Zhang et al., "Advances in mRNA Vaccines," 2.
[1443] Sanders, "Low-Dose, Single-Shot COVID-19 mRNA Vaccines Lie Ahead," 1945.
[1444] De Alwis et al., "A Single Dose," 1970.

Some of the research into saRNA vaccines has explored both varying nanoparticle formats and different routes for administering the vaccine. One murine study looked at the platforms *solid lipid nanoparticles* (SLNs), *polymeric nanoparticles* (PNPs) and *lipid nanoparticles* (LNP—both cationic LNP (cLNP) and ionizable LNP (iLNP) formats), as well as administration of vaccine via *intramuscular* (IM), *intradermal* (ID) and *intranasal* (IN) routes. In terms of formats, with IM injection, no significant differences in IgG responses among formats emerged in the first four weeks after first dose vaccination. With ID administration, the PNP format showed significantly lower responses. A second dose greatly boosted immune response (about 3-fold) with both IM and ID administration, except for the PNP format. Overall, the study found similar immunogenicity after either IM or ID administration, but lower immunogenicity following IN delivery. The strongest immunogenicity was seen with an iLNP format and IM administration.[1445]

In February, 2022, findings on a saRNA vaccine (LNP-nCoVsaRNA) tested on 192 human adult volunteers (ages 18–45; 62% male) in a Phase I trial were reported. All participants received two doses (dose levels from 1.0 μg to 10.0 μg) of vaccine, administered four weeks apart. The safety of the vaccine was comparable to that found with other mRNA vaccines (with lower doses showing very low levels of *reactogenicity* (post-vaccination reactions)). But the proportion of participants generating neutralizing antibodies did not reach a desirable level for a vaccine. Still, this trial provided valuable data for ongoing ssRNA development.[1446]

More promising results were soon forthcoming from another research endeavor. At the end of April, 2022, it was reported by Arcturus Therapeutics of San Diego that its placebo-controlled trial with more than 16,000 participants showed 55% efficacy against symptomatic COVID-19 and 95% efficacy against severe illness and death.[1447] The vaccine (ARCT-154) Phase III trial was conducted in Vietnam during the period when, first Delta, then Omicron, were the dominant variants. It employed two 5.0 μg doses. The incidence of unsolicited adverse events in the vaccinated group and the placebo group were comparable—and absent any such complications as myocarditis or pericarditis.[1448]

The hope put into ssRNA vaccines is expressed by one group of researchers who remark, "[S]elf-replicating mRNA (replicon) vaccines may offer dual advantages of protective immunity with a single low-dose administration. A single-dose vaccine would not only avoid logistics and compliance challenges associated with multi-dose vaccines but also allow vaccination of more individuals with each batch."[1449]

[1445] Anderluzzi et al., "The Role of Nanoparticle Format and Route of Administration."
[1446] Pollock et al., "Safety and Immunogenicity of a Self-Amplifying RNA Vaccine."
[1447] Cohen, "First Self-Copying mRNA Vaccine Proves Itself in Pandemic Trial," 446.
[1448] Arcturus Therapeutics, "Arcturus Announces."
[1449] De Alwis et al., "A Single Dose," 1971.

The Johnson & Johnson (J&J, formally designated *JNJ*-78436735 or Ad26.COV2.S) vaccine received emergency use authorization (EUA) by the U.S. FDA on February 27, 2021. It is a single dose vaccine—an attractive alternative to those who strongly dislike shots or prefer to get vaccination done in one visit rather than having to schedule two.

The reason the J&J vaccine requires only one shot is because it is a different kind of vaccine. It is a *viral vector vaccine*, meaning it employs a virus—the so-called 'vector'—to prompt an immune response. More completely, it is a 'replication-deficient adenovirus vectored vaccine to encode the full S protein of SARS-CoV-2.' To parse this a bit, what the vaccine does is take the gene coding for the spike (S) protein of SARS-CoV-2 and add it to the modified respiratory virus adenovirus 26. The modification prevents replication (hence 'replication-deficient' in the formal description).[1450]

Adenovirus vector vaccines have been around for decades. Such vectors are easily multiplied to high titers in cell lines, show high transduction efficiency, demonstrate high transgene expression, and possess a broad range of viral tropism (meaning they can interact with target cells via interactions facilitated by how the vector is made).[1451]

The virus used is *not* COVID-19. But it is one that tells the cells it invades to make a spike protein like that found with COVID-19. When the infected cells show this protein the immune system reacts by producing antibodies *as if* the body was facing the more serious virus. In this manner, without getting COVID-19, the body learns to recognize it and is ready if it encounters it.[1452]

How the Johnson & Johnson Vaccine Is Made and Works

As explained in the previous chapter, Johnson & Johnson's Ad26.COV2.S vaccine is a 'replication-deficient adenovirus vectored vaccine to encode full S protein.' What this means, as just noted above, is that a harmless version of another virus (the 'vector'), one that cannot replicate itself, is used to teach the immune system to recognize the spike (S) protein of SARS-CoV-2. It is sometimes called a 'Trojan horse' approach.[1453]

This vector is packaged into an injectable form and delivered by a shot into the upper arm muscle. In that muscle it enters host cells and instructs those cells by first acting like SARS-CoV-2 to cause a harmless version of a portion of the virus to be made. This is then displayed on the surface of the host cell, which attracts attention by the surveillance operation of the immune system. The alerted immune system then mounts a defense. Key to this defense are antigen-specific antibodies that now and henceforth can recognize the viral profile it has

[1450] Livingston, Malani, and Creech, "The Johnson & Johnson Vaccine for COVID-19."
[1451] Ura et al., "New Vaccine Production Platfors," 198; Chong et al., "Adenovirus Tropism."
[1452] See CDC, "Understanding Viral Vector COVID-19 Vaccines."
[1453] Samaranayake, Seneviratne, and Fakhruddin, "Coronavirus Disease 2019," 6 (Table 2).

been shown. Thus if and when the actual, *harmful* virus portion shows itself the human body is ready to spring into defensive action.

Advantages

Above we saw that one attraction of this vaccine is that *it only requires one shot*. Another is that because it is based on stable DNA molecules (whereas mRNA vaccines are based on highly unstable RNA molecules), *the vaccine does not require ultracold storage to preserve it*.[1454] The Pfizer vaccine, before mixing, initially required storage at very cold temperatures (between −90° and −60° Celsius; −130° and −76° Fahrenheit), which necessitates an extra-cold freezer. At these temperatures it can be stored until its expiration date. But the current recommendations modified what is allowed. First, the vaccine can be stored for up to 2 weeks in a freezer at more moderate temperatures (−25° and −625° C; −13° and 5° F), much like other vaccines. Second, it can be transferred from freezer storage to a refrigerator for up to 31 days at even warmer temperatures (2°–8° C; 36°–46° F). After mixing for vaccination, it can only be preserved for a 6 hour period at room temperature (2°–25° C; 35°–77° F). The Moderna vaccine requires storage between−25° C and −15° C (for up to 4 months), or 2°C–8° C (for up to 1 month)—the temperature ensured by a standard medical refrigerator.[1455]

The Active Ingredient

The formal description of the active ingredient of Johnson & Johnson's vaccine is "Recombinant, replication-incompetent Ad26 vector, encoding a stabilized variant of the SARS-CoV-2 Spike (S) protein."[1456]

There is no reason to be coy about the 'vector.' The background virus used as the base for the vector has been known and employed in vaccines for decades. It is *adenovirus type 26* (Ad26) *wild type virus*. This virus is associated with asymptomatic or mild illness. To use it as a vector the virus has to be rendered harmless, meaning unable to replicate itself while being useful to convey information to the human host cells. Thus the vector system for a replication-deficient adenovirus vectored vaccine consists of a portion of the virus—an *adaptor plasmid* to provide cloning sites without interfering with transcription or translation inside the cell, and a *cosmid*—a hybrid plasmid required for DNA packaging. Artificial transfer of this material ('transfection') into a cell line packaging, with appropriate recombination of the adaptor plasmid and cosmid results in what is termed a 'replication-incompetent vector'—something that can provoke an immune response but isn't able to replicate itself inside the host.[1457]

[1454] Livingston, Malani, and Creech, "The Johnson & Johnson Vaccine for COVID-19."
[1455] CDC, "Pfizer-BioNTech COVID-19 Vaccine" (Dec. 13, 2021); Roncati and Corsi, "Nucleoside-Modified Messenger RNA COVID-19 Vaccine," 4056.
[1456] CDC, "Johnson & Johnson's Janssen COVID-19 Vaccine Overview and Safety."
[1457] Custers et al., "Vaccines Based on Replication Incompetent Ad26 Viral Vectors," 3082. On adaptor plasmids, see Hughes et al., "Adaptor Plasmids Simplify the Insertion of Foreign DNA."

The Inactive Ingredients

Here's the list of inactive ingredients for J&J's vaccine:[1458]

Ingredient Type	Ingredient Name	Purpose
Acids/Acid Stabilizers	*Citric acid monohydrate; Trisodium citrate dihydrate*	Both are acid stabilizers used to assist in vaccine stability.
Salt	*Sodium chloride*	Assists in vaccine stability.
Sugar	*Sucrose*	Assists in vaccine stability, structural integrity of the active ingredient and ongoing vaccine effectiveness.
Other	*2-Hydroxypropyl-β-cyclodextrin* (HBCD)	Assists keeping active ingredient in stable liquid form.
	Polysorbate-80 (Tween 80)	Vaccine stabilization and emusification.
	Ethanol (alcohol)	Serves to keep the adenovirus intact.

The above represents the *complete* list of ingredients found along with the active ingredient. As with the mRNA vaccines, no adjuvants are included. Many of the inactive ingredient types (e.g., acids/acid stabilizers, salt and sugar) are of kinds similar to those in the mRNA vaccines. The first two, for example, act to balance the pH of the vaccine to match that of the human body.

But there are some important differences, too. One important difference is the absence of *lipids* in the J&J vaccine. Instead of employing lipid nanoparticles (LNPs) to deliver the S protein into the body, the adenovirus serves as the carrier. This platform utilizes the AdVac® technology previously designed by the company to combat the Ebola virus.[1459]

The ingredients listed under 'Other' also are not found in the mRNA vaccines discussed earlier. All of them (HBCD, Tween 80, and ethanol) are important to vaccine stability. Each in its own way helps prevent vaccine components from breaking down or separating. HBCD keeps things liquid, while Tween 80 keeps the mixture from separating, and ethanol keeps it from breaking down.

These are not novel ingredients but can be found in other vaccines, too.

[1458] CDC, "Johnson & Johnson's Janssen COVID-19 Vaccine: Overview and Safety."
[1459] Janssen Global Services, "Vaccine Technology" (June 17, 2022 update).

Efficacy & Safety of Johnson & Johnson Vaccine

Johnson & Johnson's Ad26.COV2.S vaccine is considered an outstanding representative of Ad26-based vaccines.

Booster Efficacy/Effectiveness

At the end of 2021 a preprint study reported on South African health care workers who had received a second (booster) shot of the Johnson & Johnson vaccine compared to unvaccinated individuals in the same managed care organization. They found vaccine effectiveness (VE) against hospitalization improved from 63% within the first two weeks after vaccination to 85% within 1–2 months. The researchers concluded their data demonstrates that a booster shot of Johnson & Johnson's vaccine following an initial shot of the same is protective against hospital admissions.[1460]

In the United States, a study of the vaccine in 10 states between December 16, 2021–March 7, 2022, specifically addressed the effectiveness of two different strategies for those whose primary vaccination had been with Johnson and Johnson's vaccine and subsequently received a booster. In the first strategy, such persons received a booster of the same vaccine; in the second, they received a mRNA booster. Both strategies were also compared to that of people who received primary vaccination with an mRNA vaccine and then a booster of the same kind. Outcomes were examined among 80,287 emergency department/urgent care (ED/UC) visits and 25,244 hospitalizations to assess vaccine effectiveness (VE). The Table below reports the findings.[1461]

Vaccine Strategy	**Vaccine Effectiveness (VE)** against laboratory-confirmed COVID-19–associated ED/UC encounters	**Vaccine Effectiveness (VE)** against laboratory-confirmed COVID-19–associated hospitalizations
J&J primary vaccination and booster	54%	67%
J&J primary vaccination; mRNA booster	79%	78%
mRNA primary vaccination and booster	83%	90%

[1460] Gray et al., "Vaccine Effectiveness Against Hospital Admission."
[1461] Natarajan et al., "Effectiveness of Homologous and Heterologous COVID-19 Booster," 495.

More research relevant to the Johnson & Johnson vaccine is reported below in the section on comparative studies. In sum, those studies suggest that while the Johnson & Johnson vaccine is effective it may be somewhat less so than the mRNA vaccines.

Reception of the Vaccine

Altogether, midway through 2022, Johnson and Johnson's vaccine accounted for less than 1% of U.S. vaccinations. As might be expected, a number of factors can be attributed to this outcome. In addition to the success of the mRNA vaccines, there was also some alarm over reported severe adverse events that resulted in a short pause in use, and some concerns raised on moral grounds.

Religious Concerns

In part the lukewarm reception of the J&J vaccine has been due to the pronouncement by some religious leaders (e.g., Roman Catholic officials) that because of ties to a history of fetal cell line use in the background of the vaccine (it used the Per.C6 cell line originating from a fetus aborted in 1985 in its manufacturing process), the other authorized vaccines should be preferred (but if J&J's vaccine was the only one available it was allowed by the Catholic hierarchy).

The U.S. Conference of Bishops, in a document released in December, 2020, addressed three vaccines—the two mRNA ones and AstraZeneca's (widely used in Europe). Following guidance from the leadership in Rome, the Bishops pointed out that neither the Moderna nor Pfizer vaccines had any connection to 'morally compromised cell lines' in their design, development or production. Their only connection to such lines was in using the HEK293 cell line in a confirmatory test of the vaccine. This, the Bishops observe, is a very remote connection to abortion. They concluded, "In view of the gravity of the current pandemic and the lack of availability of alternative vaccines, the reasons to accept the new COVID-19 vaccines from Pfizer and Moderna are sufficiently serious to justify their use, despite their remote connection to morally compromised cell lines."[1462]

A specific statement on Johnson and Johnson's vaccine came March 2, 2021. The Bishops wrote, "if one can choose among equally safe and effective COVID-19 vaccines, the vaccine with the least connection to abortion-derived cell lines should be chosen. Therefore, if one has the ability to choose a vaccine, Pfizer or Moderna's vaccines should be chosen over Johnson & Johnson's." This recommendation was offered because the J&J vaccine "was developed, tested and is produced with abortion-derived cell lines."[1463] Nevertheless, the bottom line remained that the good of vaccination—even with the Johnson and Johnson vaccine—outweighed any evil attached to its origin and manufacture.

[1462] Rhoades and Naumann, "Moral Considerations Regarding the New COVID-19 Vaccines," 5.
[1463] United States Conference of Catholic Bishops, "U.S. Bishop Chairmen."

An Abundance of Caution

Anyone who has attended media reports on the pandemic is aware that Johnson and Johnson's vaccine has had its ups-and-downs. Here is a simple time line:

Feb. 27, 2021	Apr. 13, 2021	Apr. 23, 2021	July, 2021	Dec. 16, 2021
EUA by FDA	Pause after reports of serious adverse events	Pause lifted	Review concludes benefits outweigh risks	Recommendation mRNA boosters be preferred over J&J vaccine

Despite the good record of Johnson and Johnson's vaccine, an 'abundance of caution' led to the recommendation by the Advisory Committee on Immunization Practices (ACIP) to temporarily suspend administration after a few incidences of blood clots with low platelets became associated with its vaccination use. Out of some 6,800,000 doses given to people in the U.S., *6* cases (all women between ages 18–48) were reported. The symptoms arose in a time frame of 6–13 days after vaccination, within the window of time when a plausible suspicion could arise that the adverse event was related to the vaccination.[1464] After further review, the FDA concluded the vaccine's use should be resumed, that it is safe and effective, that known and potential benefits outweigh known and potential risks, and that the chance of adverse events such as occasioned the pause is very low.[1465]

In mid-December, 2021 (and reaffirmed in the Spring of 2022), the CDC recommended that all persons age 18 or older receive a booster shot in addition to their completed schedule of vaccination. For those who had received Johnson and Johnson's vaccine, it was advised such a booster be two or more months after the initial shot *and* that it be with a mRNA vaccine. The recommendation was because, as the CDC put it, "the risks for rare but serious adverse events following receipt of a Janssen vaccine, including thrombosis with thrombocytopenia syndrome and Guillain-Barré syndrome."[1466] *Thrombosis*—discussed in the chapter on COVID-19—refers to blood clots that block arteries or veins; *vaccine-induced thrombocytopenia syndrome* (VITT) is a condition requiring meeting five diagnostic criteria: [1] vaccine 4–42 days before symptom onset; [2] venous or arterial thrombosis; [3] a low platelet count (< 150 x 10^9/L; *thrombocytopenia*); [4] a positive on a specific serologic lab test (PF4 "HIT" (heparin-induced thrombocytopenia) ELISA); and [5] elevated D-dimer (>4x).[1467]

[1464] Oliver et al., "Use of the Janssen (Johnson & Johnson) COVID-19 Vaccine," 90; Mahase, "COVID-19: US Suspends Johnson and Johnson Vaccine Rollout."
[1465] FDA, "FDA and CDC Lift Recommended Pause."
[1466] Natarajan et al., "Effectiveness of Homologous and Heterologous COVID-19 Booster," 495.
[1467] ASH, "Vaccine-Induced Immune Thrombotic Thrombocytopenia."

Comparative Studies

A number of comparative studies have been done both between the mRNA vaccines and between them and other vaccines. Obviously, it is impossible to cover all of them even in a volume as long as this one. However, since people often find not-knowing which one is 'best' contributes to their espoused vaccine hesitancy, some coverage is desirable. Here are a dozen reports.

Study 1: AstraZeneca, Curevac, Janssen, Moderna, Novavax, Pfizer, Valneva

A 2021 study of third booster vaccination against COVID-19, titled COV-BOOST, entailed a multicentre (18 United Kingdom sites), randomized phase 2 trial involving 2,878 adult participants (all age 30 or older, mostly White, but a generally balanced Male/Female ratio). Individuals were classified as 'primed' (i.e., having received prior 2-shot vaccination) by either the AstraZeneca vaccine or the Pfizer/BioNTech vaccine. All were at least 84 days past the second shot. The participants were then administered a third—booster—shot, with one or another of seven vaccines (AstraZeneca, Curevac, Janssen, Moderna, Novavax, Pfizer, or Valneva), or of one of three of these administered at half-dose (AstraZeneca, Pfizer/BioNTech, or Valneva). Vaccine effectiveness was assessed in terms of *immunogenicity*—the ability of the vaccine to provoke an immune system response as seen in levels (titres) of neutralizing antibodies. Here are some of the results:

- Those in both the AstraZeneca-primed group and the Pfizer/BioNTech-primed group showed significantly higher levels of these antibodies (anti-spike IgG) at 28 days after the booster shot.
- All the vaccines used as a booster for the AstraZeneca-primed group boosted immunity, and all but the Valneva booster did so for members of the Pfizer/BioNTech-primed group.
- The mRNA vaccines (Moderna and Pfizer) and the Johnson & Johnson vaccine worked better as a booster with both primed groups than did AstraZeneca's vaccine as a booster (as measured by cellular responses).
- Half-dose Pfizer/BioNTech booster shots worked well-enough to suggest a higher dose may be unnecessary for a booster shot.
- All seven vaccines demonstrated acceptable levels of side-effects (the most common being pain at the injection site, headache and fatigue within 1 week).
- The Moderna vaccine was the mRNA vaccine most likely to produce side-effects (i.e., was the most *reactogenic*).[1468]

In sum, the tested vaccines served effectively as booster shots even when ones were used that were different from the vaccine received in the first two shots.

[1468] Munro et al., "Safety and Immunogenicity of Seven COVID-19 Vaccines."

Study 2: AstraZeneca, Pfizer, Sinopharm, Sputnik V

A study conducted by Naranjargal J. Dashdorj and colleagues examined antibody response to four different vaccines (AstraZeneca, Pfizer/BioNTech, Sinopharm, and Sputnik V). From July 3–7, 2021, the study gathered samples from 196 participants in Mongolia, where 97% of those with COVID-19 at the time were infected by the Alpha variant. The researchers particularly were interested in ACE2-blocking antibody activity. When the four vaccines were compared, the Pfizer vaccine produced the highest effect (followed, in order, by AstraZeneca, Sputnik V, and Sinopharm). They also found that even with breakthrough infections, all of the vaccines resulted for most individuals in elevated ACE2-blocking antibody activity.[1469]

Study 3: AstraZeneca, Johnson & Johnson, Moderna, Pfizer

At the end of 2021 a study was conducted to compare vaccine effectiveness (VE) among four vaccines. The 400 participants had received either AstraZeneca (26), Johnson & Johnson (75), Moderna (199), or Pfizer (100) vaccine. In terms of VE for neutralizing antibodies 28 days post-vaccination these ranked as follows (highest VE to lowest):

- Moderna;
- Pfizer;
- AstraZeneca;
- Johnson & Johnson.

It found for the latter two vaccines—both mRNA vaccines—that binding antibody levels against Wild Type SARS-CoV-2 were evident a month (28 days) after vaccination, but decreased significantly after 6 months. The AstraZeneca vaccine—like Johnson & Johnson's an adenovirus vector-based vaccine—showed lower initial binding antibody levels and later decrease. By contrast, though initial antibody levels with Johnson & Johnson's vaccine were lower than for the mRNA vaccines, they did not significantly decrease over a 6 month period. The vaccines performed well against Beta and Delta variants, but not as well against Omicron. A booster shot of the Pfizer vaccine after initial vaccination by any of the three vaccines approved in the U.S. partially restored neutralization of Omicron. However, in examining a subset—60 health care workers, with 15 members from each vaccine group—Spike-specific T-cell responses for all the vaccines remained good, with the strongest such responses found for those vaccinated with Moderna's mRNA vaccine.[1470]

[1469] Dashdorj et al., "Direct Comparison of Antibody Responses."
[1470] GeurtsvanKessel et al. "Divergent SARS-CoV-2 Omicron-Specific T- and B-Cell Responses."

Study 4: AstraZeneca, Moderna, Pfizer

The emergence of the Omicron variant in late 2021 raised significant concerns over vaccine effectiveness. A number of studies (reported earlier in this volume at the end of chapter 3) indicated reduced efficacy of vaccines. A study released by the United Kingdom Health Security Agency of Omicron cases from November 27 (when it was first identified in the UK) through December 20, 2021, examined vaccine effectiveness (VE) against symptomatic disease. No matter how assessed, effectiveness against Omicron was reduced compared to against Delta. It found, however, variable results for booster shots after one or another of either AstraZeneca or Pfizer vaccines for original vaccination. The results reported were:

- Original: Pfizer
  - Booster: Moderna (70% VE 2–4 weeks later; 70–75% up to 9 weeks)
  - Booster: Pfizer (70% VE 2–4 weeks later; 45% after 10 weeks)
- Original: AstraZeneca
  - Booster: Moderna (60% VE 2–4 weeks later; 45% after 10 weeks)
  - Booster: Pfizer (60% VE 2–4 weeks later; 35% after 10 weeks)

Those who had been vaccinated with Pfizer's vaccine, then boosted with either Pfizer or Moderna did relatively better than those vaccinated originally with AstraZeneca.[1471]

Study 5: Johnson & Johnson, Moderna, Pfizer

An 'interim report' in the *Morbidity and Mortality Weekly Report* in the summer of 2021 examined data collected from 32,867 medical encounters across 187 hospitals and 221 emergency departments and urgent care clinics in 9 states during June–August. All cases were of individuals who had received SARS-CoV-2 testing and had received a diagnosis of a COVID-19-like illness. Overall, vaccination effectiveness (VE) against hospitalization was 86%. Across all ages, VE was reported as follows:

- Moderna: 95% VE against hospitalization;
- Pfizer: 77% VE against hospitalization; and,
- Johnson & Johnson: 65% VE against hospitalization.

These results, as the report indicates, were during the period when Delta was the dominant variant for SARS-CoV-2.[1472]

[1471] UK Health Security Agency, "SARS-CoV-2 Variants" (Technical Briefing 33), 25, and see Fig. 10 (p. 26).

Study 6: Johnson & Johnson, Moderna, Pfizer

In an examination of outcomes for 1,228,664 completely vaccinated persons whose information was in the Premier Healthcare Database Special COVID-19 Release (PHD-SR) for the period December 2020–October 2021, those who received the Moderna vaccine had a somewhat better adjusted odds ratio (aOR) with respect to a severe outcome if infected by SARS-CoV-2. Those who received either the Johnson and Johnson vaccine or the Pfizer vaccine had a similar aOR for severe COVID-19 (aOR 0.70), compared to Moderna's aOR (0.56). This meant those who received the Moderna vaccine had a 44% lower risk whereas those receiving either of the other vaccines had about a 30% lower risk of severe disease.[1473]

Study 7: Johnson & Johnson, Moderna, Pfizer

A comprehensive review of the three authorized U.S. vaccines, published in 2022, reported that clinical trials found both Moderna and Pfizer vaccines at about 95% efficacy, and the Johnson and Johnson vaccine at 66% efficacy in protecting against moderate and symptomatic infection by SARS-CoV-2. Despite noting the 'significant' difference in efficacy between the two mRNA vaccines on one side and Johnson and Johnson's adenovirus-vectored vaccine on the other, the review concluded all three possess "very high effectiveness in preventing hospitalization and death."[1474]

Study 8: Johnson & Johnson, Moderna, Pfizer

In at least one manner, expression of public sentiment about the different vaccines from late 2020 into the Spring of 2021 showed some correspondence to what scientific data was showing about the relative effectiveness of the three approved vaccines. A study of more than a million Twitter tweets found Moderna's vaccine receiving the most positive response, followed by Pfizer's and then Johnson and Johnson's vaccine. Not surprisingly, then, the last named also received the most negative remarks and Moderna's the fewest. None of the vaccines achieved even 50% positive comments, though all three had more positive sentiment than negative.[1475]

Study 9: Moderna, Pfizer

In the fall of 2021, when the Delta variant was dominant in the U.S., a study utilizing data from the VISION Network[1476] found that the risk of hospi-

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[1472] Grannis et al., "Interim Estimates of COVID-19 Vaccine Effectivness."
[1473] Yek et al., "Risk Factors," 20.
[1474] Patel et al., "A Comprehensive Review of SARS-CoV-2 Vaccines." Quote from p. 10.
[1475] Huangfu et al., "COVID-19 Vaccine Tweets after Vaccine Rollout," 8.
[1476] The VISION network includes Columbia University Irving Medical Center (New York), HealthPartners (Minnesota and Wisconsin), Intermountain Healthcare (Utah), Kaiser Permanente Northern California (California), Kaiser Permanente Northwest (Oregon and Washington), Regenstrief Institute (Indiana), and University of Colorado (Colorado).

talization for COVID-19 was 5.49 times higher for those previously infected than those vaccinated who were experiencing a 'breakthrough' infection. They also reported finding that the benefit of vaccination appeared to be higher for recipients of Moderna than for those of Pfizer-BioNTech.[1477]

Study 10: Moderna, Pfizer

In late December, 2021, a Danish study of vaccine effectiveness (VE) against Omicron, based on nationwide databases, was reported in a preprint article. Researchers at Copenhagen's Statens Serum Institut studied records from the period November 20 through December 12, 2021. Specifically, they found relatively higher VE for Pfizer than for Moderna:

- Moderna, original 2-shot vaccination—36.7% VE after 1 month; rapidly wanes.
- Pfizer, original 2-shot vaccination—55.2% VE after 1 month; rapidly wanes; with booster, returned to 54.6% VE (studied only with those age 60 or older).

The study found both vaccines showed lower VE against Omicron than against Delta. However, the authors conclude by emphasizing that while original vaccination's protection quickly fades against Omicron, a booster shot significantly increases protection.[1478]

Study 11: Moderna, Pfizer

An article in *The Scientist* published in late September, 2021, tried to summarize a number of studies to answer the question which of the two mRNA vaccines might be 'better.' Although early data showed the two vaccines to be highly comparable in performance, over time some divergences began to show. Perhaps most notable was the greater protection offered against breakthrough infections by Moderna's vaccine—a two-fold reduction in risk. Another area where Moderna's vaccine seemed to outperform Pfizer's was in preventing hospitalizations (Moderna 95% effective; Pfizer 80%), and preventing emergency room visits or urgent care (Moderna 92% effective; Pfizer 77%). Finally, in terms of robust antibody response, those receiving Moderna's vaccine showed significantly higher concentrations of spike-specific antibodies 6–10 weeks after completing their vaccination schedule.[1479]

Study 12: Johnson & Johnson, Pfizer

At the end of 2021 a study from South Africa reported in preprint examined T-cell response to Omicron among different groups, including those vaccinated by Pfizer's vaccine and Johnson & Johnson's vaccine. It examined 20 participants who had received this vaccine then contracted the emerging Omi-

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[1477] Bozio et al., "Laboratory-Confirmed COVID-19."
[1478] Hansen et al., "Vaccine Effectiveness."
[1479] Manjarrez, "Moderna vs. Pfizer: Is There a 'Best' mRNA Vaccine?"

cron variant. It found a good preservation of T-cell response against Omicron—one comparable to earlier variants like Beta and Delta. Still, even with a second (booster) shot, those vaccinated with Johnson & Johnson's vaccine showed a significant decrease in the magnitude of Omicron spike-specific CD8 T-cells, something not true for those who received the Pfizer vaccine. The 15 participants who had received full (2 dose) vaccination of the Pfizer vaccine showed 70-80% of T-cell response was preserved. Thus, overall, even though Omicron shows greater escape against neutralizing antibodies it fares no better against T-cells than did earlier variants like Beta and Delta. The study's authors conclude, "The limited effect of Omicron's mutations on the T cell response suggests that vaccination or prior infection may still provide substantial protection from severe disease."[1480]

Other Research

After the mRNA vaccines were approved for use with younger people (ages 12–17) the Pfizer vaccine became more popular than the Moderna one for that age group because of a concern over a possible side-effect of myocarditis. By mid-November of 2021, some nations (e.g., Denmark, Sweden) had halted administration of the Moderna vaccine for this age group. Others (e.g., Germany) simply recommended Pfizer in preference to Moderna.[1481]

Booster Shots

Although in the United States the Pfizer vaccine into June, 2022, had been administered the most for first vaccine dose (60.9% to Moderna's 39%), the numbers tell a different story for booster doses. The margins were close for a first booster dose (Pfizer, 55.77%; Moderna, 42.75%), and even tighter for a second booster dose (Pfizer, 51.99%; Modern, 47.9%)—a difference in the latter of only about 4%.[1482] However one might attribute the changes in American preference for whose vaccine they received, one fact remained clear: mRNA vaccines accounted for some 99% or more of them.

For both the mRNA vaccines considered in this chapter, and for AstraZeneca, booster shots have been demonstrated to significantly boost neutralizing antibody titres[1483]—an established measure for assessing vaccine effectiveness. By early 2022, some vaccine candidates were aiming specifically at functioning as booster shots, such as the Sanofi-GSK vaccine, which reported in its phase 3 trials that it increased antibody levels by 18- to 30-fold and was 100% effective against severe disease and death.[1484]

[1480] Keeton et al., "SARS-CoV-2 Sike T Cell Responses." Quote is from p. 4.
[1481] Owusu, "Germany Recommends Pfizer/BioNTech Vaccine Over Moderna's."
[1482] CDC, "COVID Data Tracker" (June 6, 2022; https://covid.cdc.gov/covid-data-tracker/#vaccinations_vacc-total-admin-rate-total.)
[1483] Munro et al., "Safety and Immunogenicity of Seven COVID-19 Vaccines," 2 {Research in Context box].
[1484] Mandavilli, "A New Covid Vaccine." Also see Sanofi, "Update on Our COVID-19 Vaccine Candidate" (https://www.sanofi.com/en/our-covid-19-vaccine-candidates).

The French Directorate of Research, Studies, Evaluation and Statistics (*La Direction de la recherche, des études, de l'évaluation et des statistiques* (DREES)), under the auspices of the Ministry of Solidarity and Health (*Ministère des Solidarités et de la Santé*), issued a report at the end of January, 2022. In it they noted that getting a booster shot "very significantly improves vaccine protection for risks of death, entry into critical care, conventional hospitalization and infection."[1485]

A preprint study released in early 2022 reported that while a booster shot produces less robust protection against infection, it offers high protection against serious illness. Moreover, the booster shot's protection is accompanied by an increase in memory B cells specific to the receptor binding domain of SARS-CoV-2, thus forming an effective defense against future infection, *and* the antibodies encoded by these cells demonstrate increased potency and develop a diverse memory B-cell repertoire able to respond rapidly and effectively to variants.[1486]

Next Generation Vaccines

The previous chapter in its section on coronavirus vaccines offered a brief glimpse at the various avenues of approach being pursued against SARS-CoV-2. As this volume was being prepared the first urgent need to provide safe and effective vaccines had been met. This provided scientists the luxury of turning attention to both improving what is available and developing alternatives offering different features that might prove particularly desirable to one or another group (e.g., nasal spray vaccination for those fearful of shots). While much of the attention was on fine-tuning vaccines against emerging variants, especially to provide booster shots for those already vaccinated, there also were efforts to think ahead to next generation vaccines against COVID-19.

The holy grail of a next generation vaccine only starts with a desire for providing strong protection against infection "by all known and any future SARS-CoV-2 variants." That kind of effort has started already. But the vision driving some researchers—a 'dream' vaccine—is one capable of effectively protecting against other coronaviruses, especially the betacoronavirus branch of the family that includes SARS and MERS.[1487]

Developing such a vaccine may be comparable to scientists in the early 1960s considering how to put a man on the moon. And yet the task was accomplished within a decade. A similar plan, using incremental steps, can yield comparable success. But there must be the will and the support to sustain such an endeavor. That remains an open question and one that leads to our final chapter.

[1485] DREES, "Le Variant Omicron," 14: *"améliore très significativement la protection vaccinale pour les risques de décès, d'entrée en soins critiques, d'hospitalisation conventionnelle et d'infection"* (And see Table 1, p. 16).

[1486] Muecksch et al., "Increased Potency and Breadth of SARS-CoV-2 Neutralizing Antibodies."

[1487] Tan et al., "Pan-Sarbecovirus Neutralizing Antibodies," 1405.

Chapter 9
Epilogue: Lessons from a Pandemic?

Perhaps the most important lesson to learn from this pandemic is that if we *don't* learn from it we will surely pay a stiffer price in the next one. For there will be more pandemics. Coronaviruses have already yielded three highly serious diseases in the last quarter-century, and there is no sign that SARS-CoV-2 is finished with us, let alone any new coronavirus that emerges from the wild. Viruses as a group remain significantly unknown. Researchers Pasquale Campagna and colleagues remind us, "Noteworthy, it has been estimated that several hundred thousand different viruses exist and they encode a huge number of proteins, which are mostly absent in the available protein databases and perform completely unknown functions."[1488] We have much to learn yet, and alongside the vigor of scientists is needed a public support for reliable knowledge over dangerous and harmful beliefs.

It is challenging to write a book with the thought that the people who might profit the most from it are the least likely to read it. Yet the sad reality in the current United States is that we are plagued by a Culture of Belief that has succeeded in replacing confidence in science with fear of it, substituted authoritarianism for reason and evidence, and championed naive credulity nurtured in anxiety and fear. The irrational and often both politically sustained and profit-motivated anti-vaccine movement has utilized the power of fear-mongering to undermine public health initiatives.

The Anti-Vaccine Movement ('Anti-Vaxxers')

How successful have anti-vaxxers been? In early November, 2021, the Kaiser Family Foundation (KFF) presented to American adults eight false statements set out by anti-vaxxers and found that nearly two-thirds (64%) of those exposed to these falsehoods could not identify them as such. In fact, more than three-quarters (78%) of Americans either were unsure about or actually believed at least one of the eight untrue statements.

The most often endorsed erroneous statement is that the government exaggerates the number of deaths attributed to COVID-19—a sentiment that plays into a more generally cultivated mistrust of Big Government promoted by political conservatives. Similarly, the third most endorsed falsehood is that the government is also intentionally hiding the number of deaths due to vaccines for COVID-19.

Underscoring the sense that political ideology helps fuel the anti-vaccine movement, a whopping 94% of Republicans were 'unsure about' or 'believe' at least one anti-vax falsehood. Republicans were more than three times as likely as Democrats to endorse or at least entertain the veracity of at least half of the lies.

[1488] Campana et al., "Dendritic Cells and SARS-CoV-2 Infection," 2.

Altogether, nearly one-half (46%) of those identifying as Republicans were this highly credulous.

The statements tested on the public by KFF include very familiar anti-vax claims intended to undermine public confidence, such as both that the vaccine can cause infertility and pregnant women should not get it, as well as the nonsense that vaccines contain a microchip and can alter one's DNA. Because anti-vaxxers profit from touting alternatives another of the statements is the claim that Ivermectin is a safe and effective treatment for COVID-19.[1489]

Clearly my earlier book examining arguments about COVID-19 vaccines has not been read enough.

Why have the anti-vaxxers been so successful? They have, unwittingly or not, learned the ways of a virus. Profit-motivated anti-vax leaders flame anxiety into active fear with a simple message that pre-empts key cultural values and tricks followers into mindlessly replicating that message, which undergoes mutations, some of which further the spread of the viral untruths. Like the coronavirus, which attaches itself to a host and then subverts key processes in the host, anti-vaxxers use widely shared cultural beliefs to hijack normal reasoning in people, subverting their thinking through viral fear, replicating a false message that infects others spreading a harmful, diseased belief.

What is that viral belief message? Boiled down to its essentials the anti-vax belief packet is this:

- COVID-19 vaccines are more to be feared than COVID-19 itself. (This is the RNA of the anti-vaxxers).
- COVID-19 is generally mild and confers natural immunity. Natural immunity is better than vaccine immunity, which is as suspect as the vaccines themselves. COVID-19 vaccines do as much harm as—or more than—COVID-19. (This is the nucleocapsid of anti-vaxxers.)
- The government lies about COVID-19 and can't be trusted; it wants to take away individual liberties. (The anti-vax 'envelope' that comes into contact with people.)

The 'spike' on the anti-vax virus is the appeal to a fear of losing personal freedom. It gains entry into the person's belief system and proceeds to replicate its basic message. This leads distorted thinking to conclude that COVID-19 vaccines should be resisted by refusing to obey mandates or receive the vaccine.

From 'Pandemic' to 'Endemic'?

By early 2022—the third year of living with SARS-CoV-2 and COVID-19—talk was increasingly turning to recategorizing the disease as an *endemic* one. As reporter David Axe put it in a mid-February article, "Once a country or community has all the tools it thinks it needs to reduce the threat a disease poses to

[1489] Kaiser Family Foundation, "COVID-19 Misinformation Is Ubiquitous."

an acceptable level, the disease becomes endemic and people get on with their lives."[1490]

But this change was, in mid-March, 2022, still not self-evident. Indeed, at that point it looked like instead yet another wave of the pandemic might be starting. For example, in France the Directorate of Research, Studies, Evaluation and Statistics was reporting that for the week ending March 23rd, more than 2.5 million RT-PCR and antigen tests for SARS-CoV-2 were validated—the first increase in some two months in the weekly cumulative number of tests. The largest increase in testing was found for those ages 26–40, with those ages 16–25 years old not far behind.[1491]

The situation wasn't looking any better by the end of the next month. In the last week of April, CDC Director Dr. Rochelle Walensky reported that COVID-19 cases were again on the rise, having jumped 22.7% in just the previous week, to some 44,000 new cases *a day*. Hospitalizations were also on the rise—up 6.6% in the same time span.[1492]

Again in May, 2022, COVID-19 cases were on the rise in some places as the United States marked more than 1 million dead from SARS-CoV-2. And the optimistic picture that Omicron might be more infectious but was less deadly was being challenged by research suggesting its intrinsic severity might be equal to its predecessors.[1493] Serious concerns were persisting halfway through 2022.

Even *if* SARS-CoV-2 is trending toward becoming just another seasonal 'bug' it does not follow that vaccines are therefore unnecessary. Consider a hypothetical case: a virus causes death in 3% of cases (in March, 2021, the actual mortality rate for COVID-19 was estimated by WHO at 3.4%). Thus within a fixed period of time there are 100,000 cases and 300 people die. Now suppose a variant of that virus is ten times more infectious but only half as deadly; in 100,000 cases 150 people die. But instead of just 100,000 cases—because the variant is ten times more infectious—in that same fixed period of time 1 million cases occur and 225 people die. The less severe variant has caused more than twice as many deaths in the same period of time.

The Continuing Specter of Omicron

Experts certainly weren't considering the Omicron variant as knocked out even midway through 2022. WHO reported that its sub-variant B.2 was still *increasing* in number of infections; by the first part of February, 2022, it accounted for more than a fifth (21.09%) of Omicron cases, and in mid-February some ten nations (Bangladesh, Brunei Darussalam, China, Denmark, Guam, India, Montenegro, Nepal, Pakistan, and the Philippines) were reporting it was the dominant (i.e., more than 50% of documented infections) type of SARS-CoV-2 within their borders. Even in the U.S., where it remained relatively less

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[1490] Axe, "What the Hell Is Happening to COVID in Israel?"
[1491] DREES, "Semaine du 7 au 13 mars 2022."
[1492] Mishra and Steenhuysen, "More than Half of Americans Have Had COVID."
[1493] Strasser et al., "SARS-CoV-2 Omicron Variant Is as Deadly as Previous Waves."

found, it still tripled in the span between January 29th and February 5th (1.2% to 3.6%). Alarmingly, early data from nations like Denmark suggested an even greater infectiousness, being 30% more transmissible than the original strain of Omicron (B.1), which was the most infectious variant to date. Its severity appeared to be about the same as that first strain, but WHO cautioned the evidence remained limited. There was good news with respect to the performance of vaccines against it, though. They appeared to hold their own against B.2 similarly to their performance against B.1.[1494]

The B.2 sub-variant also was showing itself a threat in terms of reinfection. A preprint study released in late February, 2022, reported that of 187 reinfection cases—all from individuals with two positive samples for the virus that were more than 20 days but less than 60 days apart—47 (25%) were cases of B.2 infection after an original B.1 or Delta variant infection. These were mostly in young *unvaccinated* people. Their research suggests that this capability of BA.2 to cause reinfection in people who already have been infected by Omicron BA.1 and lack protection from vaccination may be an intrinsic B.2 property.[1495]

The Rising Specter of 'Deltacron,' BA.2, XD, XE etc.

As if the lingering threat of Omicron was not enough, in March, 2022, the World Health Organization (WHO) acknowledged it was tracking a SARS-CoV-2 variant that had been detected in Denmark, France, the Netherlands—and the United States. This new variant, circulating at least since January, 2022, was informally dubbed 'Deltacron', because it showed genetic linkage to both Delta and Omicron variants. Though not certified as a recombinant strain at the time, the emerging evidence showed the virus was not through with us yet.[1496] Deltacron was assigned Pango lineage designation 'XD.'[1497]

That seemed evident that same month in the steady rise of Omicron variant BA.2, a strain more infectious than the original Omicron (50–60% more transmissible by some estimates), and sometimes dubbed 'stealth Omicron.' In the U.S., for the week ending March 5, BA.2 accounted for 14.2% of cases; by the end of the following week it was accounting for 23.1% of all U.S. cases, and in some places substantially more (e.g., 39% of cases in New Jersey and New York). In addition to the Northeast, proportions of COVID-19 infections due to BA.2 were rapidly gaining in the West. In the lower West (e.g., California and Arizona) during that same time span BA.2 cases rose from 17.1% to 27.7%,

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[1494] WHO, "COVID-19 Weekly Epidemiological Update," 7–9.

[1495] Stegger et al., "Occurrence and Significance of Omicron BA.1 Infection Followed by BA.2 Reinfection."

[1496] For general discussion of the subject, see Snider, "There May Be a New COVID Variant." For more technical discussion, see "Delta (AY.4) and BA.1 Recombinant in France/Denmark," accessed online 3/10/2022 at https://github.com/cov-lineages/pango-designation/issues/444.

[1497] See Pango lineage list (https://cov-lineages.org/lineage_list.html) and also technical discussion (https://github.com/cov-lineages/pango-designation/issues/444).

while in the upper West (e.g., Washington and Oregon) BA.2 cases went from 16% to 26.2%.[1498]

But already the very next month, April, 2022, a new recombinant variant—*Omicron XE*—was reported to be gaining ground. This variant, first reported in the United Kingdom in mid-January, 2022, combines original Omicron and the stealth variant BA.2. It is reputedly 10% more infectious than BA.2, making it the most transmissible variant to date[1499]—until suddenly it wasn't. Before April ended, two other Omicron BA.2 sublineages—BA.2.12 and BA.2.121—were estimated to have a 23–27% growth advantage over the original BA.2 variant,[1500] and were at that time the most transmissible strain of SARS-CoV-2.

Persistent American Lassitude

Early in the pandemic it could be better understood why so many Americans were uncertain about the need to be vaccinated against COVID-19—why they were 'hesitant.' But this hesitancy persisted for many well into the thick of the pandemic. A study of 1,122,139 Twitter tweets from December 14, 2020, to April 30, 2021 divided the sentiments about vaccines into five categories and reported the following:[1501]

	Highly Positive	**Positive**	**Neutral**	**Negative**	**Highly Negative**
Response %	0.80%	46.51%	28.7%	23.81%	0.18%

Astoundingly, despite all the data readily available and the devastatingly mounting death toll, more than a quarter of people declared themselves 'neutral' and almost another quarter were 'negative' or 'highly negative.' *Less than half of the sentiments shared by Twitter were positive*. If nothing else this is an astonishing display of the failure to adequately communicate the safety, effectiveness, and benefits of vaccination. But it is also a testament to fear and ignorance.

Too many Americans remain indecisive and unprepared for the future. Scientific researcher Eric Topol, professor of molecular medicine at the Scripps Research Institute, points out, "In the U.S., there are more than 19 million children under the age 5 for whom there is no vaccine approved, and tens of millions of people have not been vaccinated. We currently rank 67th in the world for being 'fully vaccinated' and 54th for having its population get an all-important booster shot that preserves a high level of protection against hospitalizations and deaths."[1502] In the last week of April, 2022, the CDC reported that

[1498] Fallon and Snider, "New COVID Variant Is Spreading across the U.S." (Using CDC data.)
[1499] WHO, "COVID-19 Weekly Epidemiological Update (Edition 85, Mar. 29, 2022)," 6.
[1500] New York State Dept. of Health, "New York State Department of Health Announces."
[1501] Huangfu et al., "COVID-19 Vaccine Tweets after Vaccine Rollout."
[1502] Topol, "Op Ed."

75.2% of children aged 11 or younger already had been infected by SARS-CoV-2—and the news wasn't much better for those in the 12–17 age group, where 74.2% carried the antibodies showing previous infection. And for those relying on previous infection conferring adequate protection from reinfection, the CDC's Kristie Walker said, "Having infection-induced antibodies does not necessarily mean you are protected against future infection."[1503] With the number of dead Americans over one million (and with COVID-19 still having proven the third leading cause of death in the U.S. in 2021[1504]), it seems rather premature to let down one's guard—though the facts on the ground still haven't stopped millions of Americans from doing so.

The damage already done and continuing to be done by the anti-vaccination movement is incalculable in terms of losses in the economy and even far worse in lives lost or forever altered by refusal to be vaccinated. What they represent is not merely scientifically wrong, but morally reprehensible. In any sober analysis, the appeal to conservative values used to shroud and make more presentable the anti-science anti-vaccination stance is mere window dressing. It represents callous and cynical opportunism by a few profiteers to line their own pockets at the expense of people deluded into thinking anti-vaxxers actually care about them more than their public health officials do. It is time to name evil for what it is.

A Way Forward

But we must do more than merely condemn anti-vax falsehood.

The positive way forward starts with learning from the past, assessing where we stand at present, and anticipating as best we can future needs. The past has taught us that vaccines work but that poor messaging about them and a lack of sensible educational preparation for general population use has allowed the anti-vaccination movement to spread its message of fear at great cost to public health. The present reveals clear and sustainable evidence that SARS-CoV-2 and its many variants persist as a significant health issue and one complicated by vaccine hesitancy and resistance that continue to give the virus room to spread and evolve. The future in light of past and present indicates a need to shape a better message, spread it more effectively and persistently (rather than just in the midst of a crisis), and develop better and more palatable vaccines.

[1503] Mishra and Steenhuysen, "More than Half of Americans Have Had COVID."
[1504] CDC, "COVID-19 Was Third Leading Cause of Death in U.S."

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The virus 'Severe Acute Respiratory Syndrome-Coronavirus-2' (SARS-CoV-2), the cause of the disease COVID-19, has been the driving force of a world-wide pandemic. In the United States alone, more than a *million* people have died from the disease and countless numbers more have suffered illness with mild to severe symptoms. Many continue to suffer, having contracted a condition known as 'long COVID.'

Complicit with the visus in driving the disease has been a phenomenon known as 'vaccine hesitancy,' a hesitancy often stoked by fear from so-called anti-vaxxers, who spread disinformtion, myths, and outright lies while peddling their own for-profit alternatives. As a result, thousands of lives have been lost that likely would otherwise have been spared had those folk possessed better knowledge and greater confidence in science and public health officials.

This volume presents fact-based reason and evidence concerning the science of viruses, the human immune system, and vaccines. Dozens of color illustrations--many directly from scientific research publications--complement the cleatly written text. Designed to put the science of these matters into a form anyone with a desire to learn and understand can grasp, *COVID-19 Virus and Vaccines* is an indispensable guide to an important subject.